AN ABSTRACT OF THE DISSERTATION OF

Sureerat Phuvasate for the degree of Doctor of Philosophy in Food Science and Technology presented on June 3, 2014.

Title: Low-temperature Depuration and Low-temperature High Hydrostatic Pressure Processing as Post-harvest Interventions for Diminishing *Vibrio parahaemolyticus* Contamination in Oysters

Abstract approved: _____

Yi-Cheng Su

Vibrio parahaemolyticus is a leading cause of acute gastroenteritis associated with consumption of seafood, particularly raw oysters. The United States Centers for Disease Control and Prevention (CDC) estimated that 45,000 cases of *V. parahaemolyticus* infection occur each year in the U.S. A recent CDC report revealed that the incidence of *V. parahaemolyticus* infection increased 76% when comparing 2011 level to 1996-1998 level. The aim of this research was to develop low-temperature depuration and low-temperature high pressure processing (HPP) as post-harvest means for decreasing *V. parahaemolyticus* contamination in oysters for safe consumption.

Fresh Pacific oysters (*Crassostrea gigas*) were inoculated with a mixed culture (10^5 MPN/g) of five clinical *V. parahaemolyticus* strains and depurated with UV-sterilized artificial seawater in a laboratory-recirculating system at refrigeration temperatures (2, 3, 7, 10, 12.5, and 15 °C) for 4 to 6 days. Depuration of oysters at 2 or 3 °C for 4 days did not result in significant reductions ($P > 0.05$) of *V. parahaemolyticus* in the oysters. However, depuration of oysters in 30-ppt seawater at 7 to 15 ºC for 5 days decreased populations of *V. parahaemolyticus* in oysters by >3.0 log MPN/g with no loss of oysters. Further studies revealed that the efficacy of depuration in reducing *V. parahaemolyticus* in oysters was influenced by water salinity with an optimum range of 20 – 30 ppt, but not types (diploid vs triploid) or sizes of oysters. The low-temperature depuration (10 - 12.5 °C, 25 ppt) can be applied as a simple and cost-effective treatment

for reducing *V. parahaemolyticus* contamination in oysters.

Investigation of the efficacy of low-temperature HPP in inactivating *V. parahaemolyticus* was conducted with clinical and environmental strains of *V. parahaemolyticus* strains in 2% NaCl solution or oyster homogenates subjected to pressure treatments (200, 250 and 300 MPa for 5 and 10 min) at 20, 15, 5 and 1.5 °C. Inactivation of *V. parahaemolyticus* cells by HPP was greatly enhanced by lowering processing temperature from 20 to 5 and 1.5 °C. A treatment of 250 MPa for 5 min at 1.5 °C resulted in complete inactivation (>6.4 log CFU/ml) of *V. parahaemolyticus* in culture suspension and oyster homogenates. This low-temperature HPP can be applied as a post-harvest process for complete elimination of *V. parahaemolyticus* from raw oysters for safe consumption.

Studies of the mechanism of HPP for inactivating *V. parahaemolyticus* cells observed cell disruption, indicated by increased leakage of intracellular materials, after pressure treatments of 200 to 300 MPa for 5 and 10 min. Scanning electron microscopy imaging revealed a clear disruption of *V. parahaemolyticus* cells after a treatment of 300 MPa for 5 min. In addition, HPP caused protein denaturation as evidenced by decreases in protein and sulfhydryl contents of cellular proteins extracted from *V. parahaemolyticus* cells after pressure treatments. SDS-PAGE analysis of cellular proteins also demonstrated changes in protein profiles of the cells after pressure treatments. These observations suggest that inactivation of *V. parahaemolyticus* cells by HPP is associated with cell membrane damage and protein denaturation, which appear to be dependent on the strain and pressure applied in a treatment.

Low-temperature Depuration and Low-temperature High Hydrostatic
Pressure Processing as Post-harvest Interventions for Diminishing
Vibrio parahaemolyticus Contamination in Oysters

by
Sureerat Phuvasate

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented June 3, 2014
Commencement June 2014

<u>Doctor of Philosophy</u> dissertation of <u>Sureerat Phuvasate</u> presented on <u>June 3, 2014</u>

APPROVED:

_____

Major Professor, representing Food Science and Technology

_____

Head of the Department of Food Science and Technology

_____

Dean of the Graduate School

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

_____

Sureerat Phuvasate, Author

ACKNOWLEDGEMENTS

CONTRIBUTION OF AUTHORS

Dr. Ming-Hui Chen was involved with the laboratory portion of Chapter 2.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

**LOW-TEMPERATURE DEPURATION AND LOW-TEMPERATURE
HIGH HYDROSTATIC PRESSURE PROCESSING AS POST-HARVEST
INTERVENTIONS FOR DIMINISHING *Vibrio parahaemolyticus*
CONTAMINATION IN OYSTERS**

CHAPTER 1

LITERATURE REVIEW

1.1 Overview

Shellfish are filter-feeding bivalves which obtain nutrients through their water filtering activity. Therefore, shellfish can accumulate and concentrate contaminants from the surrounding water if harvested from polluted areas. Many studies have reported a variety of contaminants, including metals (Eisenberg and Topping 1984; Fang and others 2003; Jiann and Presley 1997; Presley and others 1990), hydrocarbons (Blumer and others 1970; Isobe and others 2007), viruses (Beril and others 1996; Richards 1987), bacteria (Lipp and others 2001) and parasites (Fayer and others 1998), can be accumulated in shellfish. Moreover, shellfish can be naturally contaminated with pathogens (Altekruse and others 2000; DePaola and others 1990) and toxins (FDA 2012) produced by algae in coastal environments and serve as vehicles for foodborne illnesses. Both healthy persons and people with underlying medical conditions, such as liver diseases and immune system disorders, can become ill after consuming raw and lightly cooked shellfish contaminated with pathogens (Rippey 1994). Although cooking is an effective means to eliminate bacteria in shellfish, certain viruses and toxins may not be inactivated by heating processes. Illnesses caused by norwalk-like viruses and saxitoxin have been associated with consumption of steamed shellfish (Acres and Gray 1978; Kirkland and others 1996). A study reported that rotaviruses and hepatitis A virus could survive in mussels after being steamed for 5 min (Abad and others 1997).

Outbreaks associated with shellfish consumption have been sporadically reported throughout the world. Between 1969 and 2000, 46% outbreaks linked to shellfish consumption worldwide were reported in the North America followed by 28% in Europe, 13% in Asia and 13% in the Australian continent with oysters being the leading

implicated shellfish followed by clams, mussels and cockles (Potasman and others 2002). This is probably because oysters are commonly consumed raw or partially cooked. Shellfish can be vehicles for transmission of infectious diseases which occasionally result in deaths from infections. For instance, typhoid fever was the most common disease associated with raw shellfish consumption before 1950s. Several large outbreaks of typhoid involving more than 1,500 illnesses and 150 deaths occurred in the U.S. in 1920s (Rippey 1994). It was believed that release of sewage into marine environment resulted in contamination of sewage-associated bacteria and viruses in shellfish (Allen 1899).

In the U.S., more than 400 shellfish-associated outbreaks involving 14,000 cases caused by typhoid (3,270 cases), hepatitis A (1,798 cases), norwalk virus (311 cases), and unknown agents (7,978 cases) were reported between 1898 and 1980 (Rippey 1994). Since then, bacterial agents became the leading causes (60%) of illnesses associated with molluscan shellfish consumption followed by viral agents (39%). Most of the bacterial illnesses were associated with vibrios (54%) while only small portions were associated with *Salmonella* spp. (1%) and *Shigella* spp. (5%) (Iwamoto and others 2010). The large decrease in number of typhoid-associated illnesses could be due to the establishment of National Shellfish Sanitary Program (NSSP 2011) for controlling quality and safety of shellfish and growing waters since 1925.

Vibrios are naturally-occurring organisms in marine and estuarine environments. Among them, *Vibrio cholerae*, *Vibrio vulnificus* and *Vibrio parahaemolyticus* are well-known human pathogens. In developing countries, pandemic outbreaks of cholera (characterized by severe acute watery diarrhea with a high mortality rate) caused by *V. cholerae* (O1 or O139) have been mostly linked to drinking of contaminated water (Faruque and others 1998). Ingestion of raw seafood, particularly raw oysters, as well as exposure of wounds to seawater or seafood dripping are common routes of human infections caused by *V. vulnificus* and *V. parahaemolyticus* (Drake and others 2007). Both *V. vulnificus* and *V. parahaemolyticus* have been isolated from shellfish and coastal waters with high densities during the summer months (Cook and others 2002b; Duan and Su 2005; Zimmerman and others 2007).

From 1996 to 2010, 7,700 cases of *Vibrio* infections were reported through the nationwide Cholera and Other Vibrio Illness Surveillance (COVIS) system (Newton and others 2012) (Table 1.1). Two most common reported Vibrios causing infection were *V. parahaemolyticus* and *V. vulnificus*, respectively. *V. parahaemolyticus* was rarely fatal (<1%) while *V. vulnificus* infections had a highest fatal rate of >30%. According to reported *V. vulnificus* infections in the U.S. between 1988 and 1996, the infections were more frequently linked to primary septicemia (43%) and wound infections (45%) than gastroenteritis (5%) with the rest (7%) being undetermined (Shapiro and others 1998). The primary septicemia is often the main cause of fatality among persons with liver disease or other immunocompromising conditions (CDC 1996; CDC 1993). During the same period, 554 cases of *Vibrio* gastroenteritis were reported from the U.S. Gulf of Mexico regions with *V. parahaemolyticus* being the most frequently isolated strain (31%) (Altekruse and others 2000). Symptoms of the gastroenteritis were characterized by diarrhea (95%), cramps (88%), nausea (74%), vomiting (56%), fever (53%) and bloody stool (23%).

1.2 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium occurring naturally in marine and estuarine waters and frequently isolated from a variety of seafoods (Bubb 1975; Deepanjali and others 2005; DePaola and others 1990). This foodborne pathogen can cause acute gastroenteritis characterized by nausea, abdominal cramps and diarrhea mainly through consumption of raw and undercooked seafood (Daniels and others 2000a; Rippey 1994). It is recognized as the major causative agent of food poisoning associated with seafood consumption in the United States (CDC 2006; Daniels and others 2000a) and the leading cause of food poisoning in many Asian countries (Su and others 2005; Wang and others 2007; WHO 1999). Incidence of *V. parahaemolyticus* infections have also been widely reported in South America (Cabello and others 2007; Fuenzalida and others 2006; Gil and others 2007; Leal and others 2008), Europe (Martinez-Urtaza and others 2005; Ottaviani and others 2008) and Africa (Ansaruzzaman and others 2005).

Table 1.1 Number of Vibrio infections by species, reported to Cholera and Other Vibrio Illness Surveillance (COVIS) system in 1996 - 2010 (Newton and others 2012)

Vibrio species	Number of infections (%)	Number of hospitalization (%)	Number of death (%)
Vibrio parahaemolyticus	3,460 (44.9)	714 (20.6)	24 (0.7)
Vibrio vulnificus	1,446 (18.8)	1,250 (86.4)	462 (31.9)
Vibrio alginolyticus	884 (11.5)	168 (19.0)	11 (1.2)
Vibrio cholerae non 01, non 0139	697 (9.5)	278 (39.9)	35 (5.0)
Vibrio fluvialis	394 (5.1)	156 (34.2)	11 (2.8)
Vibrio mimicus	173 (2.3)	73 (42.2)	3 (1.7)
Vibrio hollisae	121 (1.6)	67 (53.1)	1 (0.8)
V. cholera 01	40 (0.5)	22 (55.0)	2 (0.5)
Species not identified	284 (3.7)	97 (34.2)	9 (3.2)
Multiple	98 (1.3)	52 (53.1)	8 (8.2)
Other[a]	103 (1.3)	48 (46.6)	4 (3.9)
Total	7,700	2,925 (40.5)	570 (8.2)

[a] Includes *Photobacterium damselae* subsp *damselae* (formerly known as *Vibrio damselae*), *Vibrio furnissii*, *Vibrio metschnikovii*, *Vibrio cincinnatiensis*, *V. cholerae* 0139, *V. cholerae* non 01, *V. cholera* unspecified and *V. harveyi*.

Shellfish, particularly raw oysters, have often been implicated in outbreaks of *V. parahaemolyticus* infection during the summer months (Daniels and others 2000b; Lozano-León and others 2003; McLaughlin and others 2005). Ecological studies have shown a strong positive correlation between water temperature and levels of *V. parahaemolyticus* in environmental samples (Costa Sobrinho and others 2010; DePaola and others 1990; Kaneko and Colwell 1973). Differences in regional climates and post-harvest handling practices can also influence densities of *V. parahaemolyticus* in shellfish at harvest and during storage for retails. High levels (>10,000 cells/g) of *V. parahaemolyticus* have been reported in retail oysters, which are potential threats to consumers' health (Costa Sobrinho and others 2011; DePaola and others 2010; Lee and others 2008).

Most *V. parahaemolyticus* strains isolated from environments are not pathogenic to humans (Lozano-León and others 2003; Matté and others 1994; Ristori and others 2007). Pathogenic strains of *V. parahaemolyticus* representing only a small percentage (3-5%) of total *V. parahaemolyticus* and have rarely been detected in environmental samples (DePaola and others 2010; Duan and Su 2005). The pathogenicity of *V. parahaemolyticus* is mainly associated with capability of producing a thermostable direct hemolysin (TDH), which is an enzyme capable of lysing red blood cells on Wagatsuma agar referred as Kanagawa phenomenon (KP) (Nishibuchi and Kaper 1995). In addition to KP$^+$ strains, KP$^-$ strains producing TDH-related hemolysin (TRH) have also been isolated from gastroenteritis patients (Shirai and others 1990). Therefore, pathogenic strains of *V. parahaemolyticus* can now be identified by detecting genes (*tdh* and *trh*) encoding TDH and TRH. However, other virulence factors have been proposed and need to be further investigated.

1.2.1 Characteristics of disease

V. parahaemolyticus is one of the *Vibrio* species most commonly associated with human diseases. Transmission of the organism occurs through consumption of contaminated seafood or by exposure of open wounds to contaminated seawater or raw

seafood products (Levine and Griffin 1993). *V. parahaemolyticus* infection usually leads to development of self-limiting gastroenteritis. An infectious dose of the organism in seafood, particular raw shellfish, was reported to be about 10^5 CFU/g (Butt and others 2004). According to the dose-response model studied for risk assessment, a probability of gastroenteritis is estimated at 0.5 (50%), in which 50 of 100 people will become sick after having a serving of oysters containing 10^8 CFU of pathogenic *V. parahaemolyticus* (FDA 2005). Therefore, the probability of illness is thus much lower (<0.0001) at a lower level (10^{3-4}) of exposure.

According to clinical data for sporadic *V. parahaemolyticus* infections occurred in the Gulf Coast states including Florida, Alabama, Louisiana and Texas reported to the United States Centers for Disease Control and Prevention (CDC) from 1988 to 1997, the predominant illness was gastroenteritis (59%), followed by wound infections (34%) and primary septicemia (5%) (Daniels and others 2000a). Clinical symptoms observed among 202 patients with gastroenteritis included diarrhea (98%), abdominal cramps (89%), nausea (76%), vomiting (55%), fever (52%) and bloody stool (29%). Gastroenteritis caused by *V. parahaemolyticus* is characterized by observing enteric symptoms with *V. parahaemolyticus* being isolated from stool (not blood) samples of a patient without evidence of wound infections. The incubation period of *V. parahaemolyticus* infection typically ranges from 15 to 19 h after ingestion of food contaminated with the pathogen and the duration of the illness usually lasts for a few days (CDC 1998; CDC 1999). Mollusks (53%) and crustaceans (35%) have been frequently reported as vehicles for *V. parahaemolyticus* infection (Iwamoto and others 2010). It was reported that 87% of patients who developed gastroenteritis after eating raw seafood had eaten raw oysters (Altekruse and others 2000). While most *V. parahaemolyticus* infections are self-limited, severe gastroenteritis and life-threatening primary septicemia associated with raw oyster consumption can occur in people with underlying illnesses such as liver disease, diabetes and alcoholism (Daniels and others 2000a). Occasionally, cases of wound infections caused by *V. parahaemolyticus* resulting in death were associated with direct contact with seawater (CDC 2005).

1.2.2 Classification of *Vibrio parahaemolyticus*

    V. parahaemolyticus can grow at broad temperatures ranging from 5 to 44 °C and pH values ranging from 4.8-11.0 in the presence of salt (1-8% NaCl). However, optimal growth of *V. parahaemolyticus* usually occurs at 30-35 °C and pH 7.6-8.6 with a salt concentration in a medium of 2-3% NaCl. Strains of *V. parahaemolyticus* share a common flagellar (H) antigen but can be differentiated by serotyping based on somatic (O) and capsular (K) antigens. There are 12 (O) groups and 65 (K) types generating at least 76 different combinations of O and K serotypes for *V. parahaemolyticus* (BAM 2004). However, there is no known relationship between serotype and hemolytic activity because *V. parahaemolyticus* strains of same serotypes have been reported to possess either hemolytic or no hemolytic activity (Sakazaki and others 1968).

    Epidemiological investigations have indicated that serotypes of clinical *V. parahaemolyticus* isolates rarely matched those of strains isolated from incriminated foods or environmental samples. For example, *V. parahaemolyticus* strains belonging to O4:K11 serovar were recovered from patients but not in incriminated foods during three episodes of U.S. outbreaks occurred in 1971 (Molenda and others 1972). Similar findings were reported in Southern Thailand where several serovars of *V. parahaemolyticus* were isolated from seafood linked to several cases of gastroenteritis with only one isolate (O3:K6 serovar) being indistinguishable from the majority of clinical isolates obtained from the patients (Vuddhakul and others 2000).

    In addition to differentiation of *V. parahaemolyticus* strains, serotyping can be carried out to identify the origin and spread of clinical strains. *V. parahaemolyticus* infections are usually associated with diverse serovars. Among them, O3:K6 strains have been frequently involved in outbreaks worldwide. A rapid increase in the incidence of *V. parahaemolyticus* was reported in India in 1996 due to the widespread of O3:K6 strains carrying the *tdh* gene (Okuda and others 1997b). In subsequent years, *V. parahaemolyticus* isolates belonging to O3:K6 serovar caused infection in many countries including Taiwan, Japan, Korean (Wong and others 2000), USA (Daniels and others 2000b), Chile (González-Escalona and others 2005), Spain (Martinez-Urtaza and

others 2005), Mozambique (Ansaruzzaman and others 2005) and Italy (Ottaviani and others 2008).

1.3 Distribution and prevalence of *Vibrio parahaemolyticus*

The occurrence of *V. parahaemolyticus* in the marine environment and in shellfish is largely associated with water temperatures (Kelly and Stroh 1988). As filter feeders, shellfish can concentrate bacteria in the seawater to a level much higher than that in the water. Many studies have investigated prevalence of *V. parahaemolyticus* in oysters as they are commonly consumed raw and often implicated in outbreaks. A recent study reported that the mean densities of total *V. parahaemolyticus* in oysters were higher than those observed in water from Mississippi (1.9 log in oysters and 0.65 log in water) and Alabama (2.9 log in oysters and 1.18 log in water) sites (Zimmerman and others 2007).

1.3.1 Water temperature

Incidence of *V. parahaemolyticus* in the environments is known to correlate with water temperatures. Many studies have showed that *V. parahaemolyticus* was rarely detected in water until temperature exceeded 15 °C. An ecological study of *V. parahaemolyticus* in the Chesapeake Bay of Maryland observed that *V. parahaemolyticus* was not detected in water samples until the middle of April when water temperatures rose to 14 °C (Kaneko and Colwell 1973). The densities of *V. parahaemolyticus* in water increased sharply when water temperatures reached 19 °C with the highest counts (620 CFU/ml) being observed when the water temperature jumped to 31 °C in July. Similarly, *V. parahaemolyticus* was detected in water samples collected from South African Coast during summer (January to July) when water temperature ranged from 14 to 21 °C (Bubb 1975). In addition, detectable levels (up to 70 CFU/ml) of *V. parahaemolyticus* in estuarine water samples from British Columbia of Canada were reported during the summer months (from June through October) when water temperatures were ≥ 17 °C (Kelly and Stroh 1988). A study conducted in Japan reported the maximum loads of *V. parahaemolyticus* in water (550 CFU/ml) and in plankton samples (1,000 CFU/g)

collected from Kojima Bay of Okayama during the warm months when water temperatures were as high as 25 °C (Chowdhury and others 1990). *V. parahaemolyticus* in water (>14 MPN/ml) and sediments (>140 MPN/g) were also observed at a Coast of the Seto Inland Sea (close to the Kojima Bay) of Japan during the summer months with water temperatures ranging from 19.5 to 28.5 °C (Alam and others 2003).

The numbers of *V. parahaemolyticus* in raw shellfish are also known to relate to the water temperatures and can be higher in shellfish harvested at warm months. This pathogen was detected in 15% oysters harvested from two oyster-growing bays in Oregon during summer months with densities as high as 43 MPN/g when the water temperature increased to 15 °C (Duan and Su 2005). High densities of *V. parahaemolyticus* (>10,000 cells/g) have been reported in oysters harvested in Washington between June and September when water temperatures were higher than 20 °C (Baross and Liston 1970). Similarly, *V. parahaemolyticus* was detected in almost all oysters (>99%) harvested from the Southern Coastal area of Sao Paulo State in Brazil, where average water temperatures were 20 °C or higher all year round (Costa Sobrinho and others 2010; Ristori and others 2007). Although the densities of *V. parahaemolyticus* in the oysters from the region were reported as high as 4.38 log MPN/g (during winter) and 5.04 log MPN/g (from spring to fall), pathogenic *V. parahaemolyticus* was rarely detected (Costa Sobrinho and others 2010). On the contrary, pathogenic *V. parahaemolyticus* was frequently detected in various shellfish (oysters, clams and mussels) from Spanish Mediterranean Coast during July and August when water temperatures exceeded 25 °C (Roque and others 2009).

1.3.2 Geographic locations

V. parahaemolyticus infection is frequently associated with shellfish consumption in temperate regions (McLaughlin and others 2005; Cabello and others 2007; Cordova and others 2002). An investigation of prevalence of *V. parahaemolyticus* in shellfish between Northern (Antofagasta) and Southern (Puerto Montt) Chile during the summer reported detection of the bacterium in 80% of shellfish samples collected from growing sites in the South region (15 to 5,500 MPN/g) but not in the samples collected from the

North region due to a difference in temperatures of seawater between the two regions (Fuenzalida and others 2007). An investigation of *V. parahaemolyticus* in oysters in the late summer following the shellfish-associated outbreaks in Washington, Texas and New York in 1997 and 1998 (DePaola and others 2000) revealed that most oysters harvested from Galveston Bay, Texas had levels of *V. parahaemolyticus* in oysters between 100 and 1000 cells/g, while the highest level of *V. parahaemolyticus* in oysters harvested from the Atlantic Northeast was 120 cells/g. In addition, densities of *V. parahaemolyticus* in market oysters in Washington varied greatly and ranged from <3 to 46,000 cells/g. These observations indicate that oysters harvested from different regions may contain different densities of *V. parahaemolyticus* and retail oysters may have higher levels of *V. parahaemolyticus* than in oysters collected from growing areas, particularly in the summer months.

Incidence of *V. parahaemolyticus* in oysters at retails in different regions has also been investigated. *V. parahaemolyticus* (3 log MPN/g) was reported the predominant species among total vibrios (4 log MPN/g) detected in market oysters collected at Korea outlets between August and September (Lee and others 2008). About 77% of oysters sold commercially in Sao Paulo State, Brazil were positive for *V. parahaemolyticus* (<3 to 1,200 MPN/g) with a mean level of 110 MPN/g (Matté and others 1994). A recent study found *V. parahaemolyticus* in all retail oysters from the same region in Brazil with densities of *V. parahaemolyticus* being above 10^4 MPN/g in 73% of the samples tested (Costa Sobrinho and others 2011). An investigation of foodborne pathogens in retail oysters in south China reported frequent detections of *V. parahaemolyticus* in 89% of oysters analyzed with 48.4% of the samples contaminated with *V. parahaemolyticus* at levels of >100 MPN/g (Chen and others 2010). In the U.S., the levels of *V. parahaemolyticus* in retail oysters vary greatly among regions. According to a survey conducted by Cook and others (2002b) between 1998 and 1999, the majority of oysters harvested from the Pacific Coast (67.3%) and North Atlantic states (57.4%) had non-detectable *V. parahaemolyticus* with <5% of the samples containing high levels of the organism at 10^{3-4} MPN/g. However, more oysters from the Mid-Atlantic (7.8%) and Gulf

Coast states (21.6%) had *V. parahaemolyticus* at levels exceeding the acceptable limit of 10^4 MPN/g. Similarly, a recent study reported high levels ($>10^5$ MPN/g) of *V. parahaemolyticus* in 9.8% of oysters harvested from the Gulf Coast and in 7% of oysters harvested from the Mid-Atlantic Coast (DePaola and others 2010). However, only 1.7% of oysters harvested in Washington were found to contain *V. parahaemolyticus* at levels of $>10^4$ MPN/g.

1.3.3 Harvesting methods

The methods (dredging and intertidal) used for harvesting oysters can significantly influence the levels of *V. parahaemolyticus* in oysters. Oysters harvested along the U.S. and Canadian Pacific Coasts during the summer months have been involved in several *V. parahaemolyticus* outbreaks (CDC 1998; CDC 2006). This could be associated with intertidal harvest method commonly practiced by the shellfish industry in the Pacific Northwest estuaries where shellfish are regularly exposed to ambient air temperature during low tide, resulting in rapid multiplication of *V. parahaemolyticus* especially on sunny days. Increased numbers of total and pathogenic *V. parahaemolyticus* in oysters were reported at various locations in Washington after 7-consecutive day of exposure (50 min each day) to air temperature (15 °C at first exposure and 20 to 25 °C at maximum exposure) from receding tides (Nordstrom and others 2004). The average densities of total *V. parahaemolyticus* in oysters were 51 and 280 CFU/g at first and maximum exposure to air at low tides, respectively. In addition, pathogenic *V. parahaemolyticus* counts were generally lower than 10 CFU/g at first exposure but increased to as high as 160 CFU/g at the maximum exposure.

It has been predicted that the overall mean levels of *V. parahaemolyticus* in oysters harvested by the intertidal method would be greater than the levels in oysters harvested by the dredging method in both Pacific Northwest and Atlantic regions (FDA 2005). For example, the numbers of total and pathogenic *V. parahaemolyticus* (650 cells/g and 15 cells/g) in Pacific oysters harvested by the intertidal practice in summer would be higher than those (5 cells/g and <1 cell/g) in dredged Pacific oysters and

Eastern oysters (230 cells/g and <1 cell/g). The annual *V. parahaemolyticus* illnesses linked to consumption of intertidally harvested Pacific oysters were estimated to be 192 cases, which were higher than the numbers of cases estimated for Pacific (4 cases) and Eastern (15 to 19 cases) oysters harvested with the dredging practice. Thus, switching oyster harvest methods from intertidal to dredging practices could reduce incidence of *V. parahaemolyticus* infection associated with raw oyster consumption.

1.4 Outbreaks

    V. parahaemolyticus is a naturally occurring marine bacterium and can be present in a variety of seafood including raw, frozen and even cooked products through post-handling contamination. *V. parahaemolyticus* has been isolated from imported variety of seafoods, including oysters (Lee and others 2008), shrimp (Cann and others 1981; Sujeewa and others 2009), crab, lobster, fish (Wong and others 1999) and frozen seafood (Honda and others 1992) in many countries. Therefore, foodborne outbreaks caused by *V. parahaemolyticus* are more likely to link to consuming seafoods which are improperly handled, cooked and served (Wang and others 2007).

1.4.1 Asia

    V. parahaemolyticus was first identified as a cause of an acute gastroenteritis outbreak in Japan involving 273 illnesses and 20 deaths resulting from eating shirasu (semi-dried salted sardine) in 1950 (Fujino and others 1953). Since then, *V. parahaemolyticus* has been frequently associated with foodborne outbreaks in Japan. The numbers of outbreaks caused by *V. parahaemolyticus* in 1996 were 102 (5,241 cases), which increased to 160 (6,786 cases) in 1997 and to 234 (12,346 cases) in 1998 (WHO 1999). Other than in Japan, *V. parahaemolyticus* was isolated in 1970s from patients with gastroenteritis in Singapore (Lam and others 1974) and India (Chatterjee and Sen 1974) and has been recognized as a major foodborne pathogen in Asian countries. *V. parahaemolyticus* was accounted for 35.5% outbreaks caused by bacteria in Taiwan between 1986 and 1995 (Pan and others 1997) and 19.5% of foodborne outbreaks

occurred in China between 1994 and 2005 (Wang and others 2007). In Korea, incidences of *V. parahaemolyticus* food poisoning periodically occurred between June and October with a higher number of cases reported in August (17-282 cases) and September (125-450 cases) between 2003 and 2006 (Lee and others 2008).

V. parahaemolyticus infections usually occur sporadically with relatively small number of cases. However, significant increases in the incidence of *V. parahaemolyticus* infection can result from a spread of any pandemic strains. The two largest outbreaks of *V. parahaemolyticus* infections recorded in Japan (691 cases in August 1996 and 1,167 cases in July 1998) were caused by pandemic strains of serotypes O3:K6 and O1:K56 after consumption of boiled crabs and catered meals, respectively (WHO 1999).

In 1996, *V. parahaemolyticus* serovar O3:K6 was reported to be associated with a sharp increase in the number of diarrheal cases in Calcutta, India and accounted for 50-80% of all *V. parahaemolyticus* strains isolated from the diarrheal patients (Okuda and others 1997b). Since then, it has been widely spread as a cause of gastroenteritis in Japan and neighboring countries. In Vietnam, 548 cases of diarrhea caused by *V. parahaemolyticus* were reported between 1997 and 1999 (Tuyet and others 2002). In Taiwan, *V. parahaemolyticus* was involved in 45% of total bacterial foodborne outbreaks in 1995 and 61-71% in 1996-1999 (Chiou and others 2000). Su and others (2005) also reported that *V. parahaemolyticus* was the most significant organism in foodborne illness outbreaks in Northern Taiwan between 1995 and 2005, accounting for 87% of isolates from clinical specimens with a predominant serovar K6 (55.2%). Serovar O3:K6 was the most frequently isolated *V. parahaemolyticus* (87%) from diarrhea patients between December 1998 and January 1999 in Songkhla Province, Thailand (Vuddhakul and others 2000).

1.4.2 North America

V. parahaemolyticus was first recognized as an important pathogen in the U.S. in 1971 after three episodes of *V. parahaemolyticus* infections of 425 cases associated with eating cooked crab occurred in Maryland (Molenda and others 1972). The first two

outbreaks were due to cross-contamination of steamed crabs being shipped and stored with live crabs, while the third outbreak was linked to crab meats being packed in cans without further heat treatment before served. In the late summer and fall of 1981, six cases of gastroenteritis and one wound infection caused by *V. parahaemolyticus* were reported in Washington and Oregon (Nolan and others 1984). *V. parahaemolyticus* was isolated from patients suffering from gastroenteritis following consumption of raw oysters. Although *V. parahaemolyticus* infection has been sporadic, a total of 40 *V. parahaemolyticus* outbreaks in 15 states and the Guam Territories were recorded between 1973 and 1998, involving 1,064 illnesses and one death associated with seafood consumption (shrimp, shellfish, clams, lobsters and crabs) (Daniels and others 2000a).

Among seafood, shellfish (particular raw oyster) has been the most frequently linked to outbreaks of *V. parahaemolyticus* infections, especially during the summer months. In the summer of 1997, an outbreak of gastroenteritis (209 cases) occurred in the Pacific Northwest was linked to consumption of raw oysters harvested in the Washington state and the British Columbia of Canada (CDC 1998). At the same time, an outbreak of *V. parahaemolyticus* (43 cases) was recorded in the British Columbia associated with consuming oysters and seafood (Fyfe and others 1997). Between May and July of 1998, raw oysters harvested at the Galveston Bay in Texas were implicated in the largest outbreak of *V. parahaemolyticus* ever reported in the U.S. A total of 416 cases of *V. parahaemolyticus* infections were reported in 13 states with *V. parahaemolyticus* O3:K6 serovar being isolated from all 28 stool samples from patients (Daniels and others 2000b). A following outbreak of 23 confirmed cases occurred between July and September of 1998 among residents of Connecticut, New Jersey and New York who consumed oysters and clams harvested from the Long Island Sound (CDC 1999). These four major oyster-associated outbreaks in 1997 and 1998 involved more than 700 cases of illness caused by *V. parahaemolyticus* in the Pacific Northwest, Gulf Coast and Atlantic Northeast.

In the summer of 2004, the first outbreak of *V. parahaemolyticus* infection in Alaska occurred on a cruise ship where 14 passengers developed gastroenteritis after

eating raw Alaskan oysters (McLaughlin and others 2005). The implicated oysters were harvested from areas with elevated water temperature (>15°C). Epidemiological investigation of the outbreak found a high detection rate (74%) of *tdh*-positive *V. parahaemolyticus* strains in environmental samples. Another outbreak (177 cases) of *V. parahaemolyticus* infection caused by oysters harvested in Washington and British Columbia was reported in New York City, New York, Oregon and Washington in the summer of 2006 (CDC 2006). In addition, wound infection associated with *V. parahaemolyticus* was linked to two deaths among three patients in Louisiana and Mississippi hit by Hurricane Katrina in 2005 (CDC 2005). In the summer of 2012, 30 confirmed illnesses caused by *V. parahaemolyticus* associated with consumption of raw and undercooked oysters were reported in Washington (Washington State Department of Health 2012). As a result, three commercial growing areas (Totten Inlet, North Bay and Dabob Bay) were closed for the rest of summer. A total of 104 cases of *V. parahaemolyticus* infections were reported in 13 states between May and September of 2013 due to consumption of raw oysters or raw clams harvested along the Atlantic Coast (CDC 2013). Harvest areas in Virginia, Massachusetts, New York and Connecticut were temporarily closed while oysters and clams linked to illnesses were recalled.

1.4.3 South America

V. parahaemolyticus, particularly pandemic O3:K6 clone, is frequently associated with diarrhea in South American countries such as Brazil (Leal and others 2008), Peru (Gil and others 2007) and Chile (Cabello and others 2007). Among them, Chile has been struck the most by the O3:K6 pandemic clone. The first outbreak of 298 cases occurred between November 1997 and April 1998 in the Northern part of Chile (Antofagasta) (Cordova and others 2002). Traditionally, *V. parahaemolyticus* outbreaks have been more common in the Southern part of Chile. It was suggested that increases in water temperature to higher than usual as influenced by El Nino could be an environmental factor contributing to the outbreak in the Northern part of Chile. In 2004, the second raw seafood-associated outbreak affecting 1,500 cases occurred in Puerto Montt, the most

important seafood processing region (González-Escalona and others 2005). In the summer of 2005, many more cases (3,725) of acute diarrhea were reported in Puerto Montt between January and April. All the *V. parahaemolyticus* strains isolated from stool samples of 60 patients were virulent O3:K6 serovar (Cabello and others 2007). Since then, the numbers of diarrhea cases reported in Chile decreased gradually in 2006 (900 cases) and 2007 (475 cases) and the percentage of clinical cases caused by O3:K6 clone also decreased from 100% in 2006 to 73% in 2007 (Fuenzalida and others 2007; Harth and others 2009). More recently, 1,143 and 441 cases of *V. parahaemolyticus* were reported in the summer of 2008 and 2009, respectively, with *V. parahaemolyticus* O3:K6 being associated with 98% of the cases in 2008 and 64% of the cases in 2009 (García and others 2009).

1.4.4 Europe

Diseases caused by *V. parahaemolyticus* are rarely reported in Europe. However, sporadic cases of *V. parahaemolyticus* infections have been reported in Spain, France, Italy and the United Kingdom. The first report of *V. parahaemolyticus* infections in Spain involved eight cases of acute gastroenteritis associated with fish or shellfish consumption in Barcelona and Galicia between August and October of 1989 (Molero and others 1989). In 1999, raw oysters were incriminated in another diarrhea outbreak of 64 cases in Galicia between August and September with 9 hospitalizations (Lozano-León and others 2003). Virulent strains (tdh^+) of *V. parahaemolyticus* were isolated from stool samples of the outbreak patients in Galicia and the strains were indistinguishable from the strains isolated from patients in Madrid and Barcelona during the same period. In 2004, an outbreak of 80 cases caused by *V. parahaemolyticus* occurred in A Coruña of Spain, where guests attending weddings at a restaurant ate boiled crabs (Martinez-Urtaza and others 2005). *V. parahaemolyticus* serovar O3:K6 and O3:K untypable isolates carrying *tdh* gene were involved in the incidence.

Imported seafood was related to an incidence of *V. parahaemolyticus* infection in France in 1999 (Lemoine and others 1999). The outbreak involved 44 cases of

gastroenteritis associated with consumption of shrimp imported from Asia. A study of occurrence of *V. parahaemolyticus* in French domestic seafood suggested that domestic seafood could also be linked to *V. parahaemolyticus* infection because pathogenic *V. parahaemolyticus* strains were generally present in seafood (oysters, cockles and mussels) and water samples collected along the French Coast (Hervio-Heath and others 2002; Robert-Pillot and others 2004).

In Italy, shellfish was sporadically implicated in foodborne disease outbreaks and accounted for 7.1% of all incriminated foods in 1998 (Tirado and Schmidt 2000). The total number of cases involved in shellfish-associated outbreaks increased from 26 (a few outbreaks) in 1999 to 223 (26 outbreaks) in 2000 (Schmidt and Gervelmeyer 2003). It was suggested that outbreaks of food poisoning associated with consumption of shellfish were mainly due to *V. parahaemolyticus* infections. A study of *Vibrio* spp. in mussels collected at retail outlets in the Puglia region of Italy between 2001 and 2004 reported that *V. parahaemolyticus* was more frequently detected in the samples (7.8%) than *Vibrio vulnificus* (2.8%) (Normanno and others 2006). In the summer of 2007, an epidemic *V. parahaemolyticus* O3:K6 strain was first isolated from a stool sample of a diarrhea patient who ate fresh shellfish bought from a local seller (Ottaviani and others 2008).

In the United Kingdom, the first recognized outbreak of *V. parahaemolyticus* infection occurred among airline crew aboard travelling from Bangkok of Thailand to London in 1972 (Peffers and others 1973). *V. parahaemolyticus* strains, which were KP$^+$ with a serotype of O2:K3, were isolated from patients as well as raw and cooked crab meat in hors d'oeuvre prepared at Bangkok**.** In 1973, locally caught crabs were implicated in an outbreak of *V. parahaemolyticus* in Britain (Hooper and others 1974). A recent study revealed that pathogenic *V. parahaemolyticus* carrying *tdh* gene was present in 10% of shellfish surveyed in the UK between 2001 and 2006 (Wagley and others 2008).

1.4.5 Africa

Approximately 50% of the African populations have no access to safe water while 66% of the populations live without sanitation (Boschi-Pinto and others 2006). Therefore,

diarrheal diseases are major causes of mortality in children under five years of age in the region. *V. parahaemolyticus* has been an organism of importance to public health as its presence in African costal water (Bubb 1975). In an early report of 1989, *V. parahaemolyticus* was responsible for 36% of diarrheal diseases caused by *Vibrio* pathogens in Nigeria (Utsalo and others 1992). Most of the patients who developed diarrhea had a history of ingestion of seafood. In 2004, *V. parahaemolyticus* was isolated from 42 stool samples of patients who had diarrhea and vomiting (81%) with a mean age of 27 (no patient younger than 5 years of age) in Beira, Mozambique (Ansaruzzaman and others 2005). Among the isolates, *V. parahaemolyticus* serovar O3:K6 and O4:K68 were first identified as pandemic strains in the Africa continent.

1.5 Virulence factors of *Vibrio parahaemolyticus*

1.5.1 Kanagawa phenomenon

Regardless of serovars, the virulence of *V. parahaemolyticus* is related to the hemolytic activity named Kanagawa phenomenon (KP). In early works, hemolysis of *V. parahaemolyticus* strains isolated from patients and environment was characterized by observing appearance of a clear halo around colonies of *V. parahaemolyticus* on the Wagatsuma agar. Sakazaki and others (1968) reported that 96.5% of clinical strains exhibited hemolysis on the Wagatsuma agar but the activity was rarely found in environmental strains (1%). This indicates KP$^-$ strains are more prevalent in the nature, but less associated with illness. Other studies also reported that KP$^+$ strains of *V. parahaemolyticus* were rarely detected in environmental samples: 1 from 187 isolates (Miyamoto and others 1969), 4 from 2,218 isolates (Thompson and Vanderzant 1976) and none from 1,484 isolates (Ayres and Barrow 1978). Virulence of KP$^+$ *V. parahaemolyticus* strains and its purified toxin have been studied in animal and human trials. Hemolysis caused by KP$^+$ *V. parahaemolyticus* was observed in infant rabbits when administrated orally (Calia and Johnson 1975). The KP$^+$ strains were able to penetrate intestinal epithelium of the rabbits and stimulate the activity while no bacteremia was observed in KP$^-$-treated rabbits. This was similar to findings of human

volunteers feeding tests conducted by Sakazaki and others (1968) who reported no sign of gastroenteritis after various serotypes of non-hemolytic *V. parahaemolyticus* (10^{6-10} cells) were administered. The investigators also reported a case of *V. parahaemolyticus* infection in a laboratory where about 10^6 cells of KP$^+$ *V. parahaemolyticus* O2:K3 was accidentally ingested. Another study observed that a purified toxin obtained from a KP$^+$ strain caused a lethal effect in mice injected with a dose of 5 μg (Honda and others 1976). Miyamoto and others (1980) also reported that a 50% of suckling mice developed diarrhea within 12 h or died within 18 h after treatments of 6 and 30 μg of the purified toxin, respectively.

1.5.2 Thermostable direct hemolysin (TDH)

The hemolysin produced by KP$^+$ strains is known as thermostable direct hemolysin (TDH) because it can not be inactivated by heating at 100 °C for 10 min (Nishibuchi and Kaper 1995). Clinical studies have revealed that nearly all *V. parahaemolyticus* strains isolated from patients with gastroenteritis demonstrated the hemolytic activity, whereas environmental isolates were rarely KP-positive. Therefore, TDH has been recognized an important virulence factor of *V. parahaemolyticus* and the gene encoding TDH (*tdh*) is commonly used as a marker for identification of virulent strains (Okuda and others 1997a; Cook and others 2002a).

The TDH consists of 165 amino acid residues with a molecular weight of approximately 42,000 daltons. It can damage erythrocyte membrane by acting as a pore-forming toxin, which alters ion flux in intestinal cells and leads to a secretary response and diarrhea (Zhang and Austin 2005). The gene encoding TDH (*tdh*) has been cloned and sequenced (Nishibuchi and Kaper 1985). The oligonucleotide probes targeting the *tdh* gene have been developed for the detection of KP$^+$ *V. parahaemolyticus* (Kaper and others 1984; Nishibuchi and others 1985).

1.5.3 Thermostable direct hemolysin related hemolysin (TRH)

Despite epidemiological investigations revealed a strong tie between the Kanagawa phenomenon and pathogenicity of *V. parahaemolyticus*, KP⁻ strains were also found to associate with gastroenteritis (Miyamoto and others 1969; Shirai and others 1990). A KP⁻ *V. parahaemolyticus* strain was isolated from an outbreak of gastroenteritis in the Republic of Maldives in 1985 (Honda and others 1988). The isolate did not carry the *tdh* gene but produced a TDH-related hemolysin (TRH), which indicated that TRH was another virulence factor of *V. parahaemolyticus*. The amino acid sequence of the TRH was reported to be 67% identical to that of the TDH and the activity of TRH could be destroyed at 60 °C or higher for 10 min (Honda and Iida 1993). The virulence of *V. parahaemolyticus* are now known to associate with the ability of producing TDH or TRH encoded by *tdh* or *trh* gene, respectively. Both TDH and TRH could induce chloride secretion in human colonic epithelial cells (Takahashi and others 2000b; Takahashi and others 2000a).

1.5.4 Other virulence factors

Most of the *V. parahaemolyticus* strains reported before 1974 were negative for urease activity (Okuda and others 1997a; Sakazaki and others 1963). However, all KP⁺ strains recovered later from patients were able to produce urease and hydrolyzed urea (Nolan and others 1984). A subsequent investigation also reported a high incidence (71%) of urease-producing strains among clinical *V. parahaemolyticus* (KP⁺) strains (Abbott and others 1989). Further studies of the virulence factors and urease production revealed that *V. parahaemolyticus* isolates associated with diarrhea were either KP⁻ with urease-positive or KP⁺ with urease-negative (Kelly and Stroh 1989). However, Osawa and others (1996) reported that 100 of 106 (94%) clinical and environmental (suspected food items) strains carrying *tdh* gene were urease-negative while all 5 *trh*-carrying strains were urease positive. The association of urease production with the *trh* gene has been demonstrated (Suthienkul and others 1995; Okuda and others 1997a). The urease (*ure*)

gene and *trh* gene were found to be genetically linked and closely localized (<8.5 kb) on a chromosome (Iida and others 1997; Iida and others 1998).

In addition to urease production, adhesiveness has been reported to play an important role in pathogenicity of *V. parahaemolyticus.* All clinical and environmental strains of *V. parahaemolyticus* showed different degree of adherence in human fetal intestine (HFI) cells (Hackney and others 1980). Clinical (KP$^+$ or KP$^-$ strains) and KP$^+$ environmental isolates were observed to have much faster and stronger adherence to HFI cells than KP$^-$ environmental strains. Baffone and others (2001) investigated several potential virulence factors (including enzymatic and biological activities and enteropathogenicity in mice) in *V. parahaemolyticus* isolated from seawater and reported that six strains of *V. parahaemolyticus* causing diarrhea and death in orally-treated mice had adhesiveness and cytotoxicity activity. In addition, all lethal strains also exhibited lipase and gelatinase activity. However, only one and three strains of them were able to produce hemolysin and urease, respectively. This indicates that pathogenicity of *V. parahaemolyticus* may be a result of a combination of virulence factors.

1.6 Detection of *Vibrio parahaemolyticus*

1.6.1 Most probable number (MPN) method

The Most Probable Number (MPN) method described in the U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM 2004) is commonly used for enumeration of *V. parahaemolyticus* in foods. This method involves enrichment of samples in alkaline peptone water (APW) followed by isolation of vibrios on thiosulfate-citrate-bile salts-sucrose agar (TCBS) plates. Briefly, 10-folded serial dilutions of a sample prepared using phosphate-buffered saline (PBS) were inoculated in APW tubes (3 or 5 tubes each dilution) and incubated at 37 °C for 16 - 18 h. Positive (turbid) APW tubes are then streaked on TCBS plates and incubated at 37 °C for 16 - 18 h. *V. parahaemolyticus*, a non sucrose fermenter, appears as round (2 - 3 mm in diameter), opaque, green to blue colonies on a TCBS plate. However, *V. vulnificus* and *V. mimicus* also do not ferment sucrose, producing similar green or bluish colonies on TCBS plates.

To differentiate *V. parahaemolyticus* from other vibrios, 3 or more typical colonies are subjected to biochemical tests. The MPN method followed by biochemical tests is labor-intensive and time consuming. An alternative to biochemical tests for confirmation of *V. parahaemolyticus* is by polymerase chain reaction (PCR) or DNA hybridization (BAM 2004).

1.6.2 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) has been developed for specific detection of the *tdh* and *trh* genes encoding TDH and TRH, respectively, of virulent strains of *V. parahaemolyticus* (Tada and others 1992).. Karunasagar and others (1996) developed a highly sensitive *tdh*-based PCR assay to detect <10 cells of *V. parahaemolyticus* in fish homogenates after 8 h of enrichment of samples in APW. However, PCR methods targeting *tdh* or/and *trh* can not be used to detect non-pathogenic *V. parahaemolyticus* strains which do not carry these genes.

For the detection of total *V. parahaemolyticus*, a PCR assay targeting *tl* gene encoding a thermolabile hemolysin (TLH) carried by all *V. parahaemolyticus* strains can be used (Taniguchi and others 1986; Taniguchi and others 1985). Bej and others (1999) developed a multiplex PCR targeting the *tl* genes for total *V. parahaemolyticus* and both *tdh* and *trh* for pathogenic *V. parahaemolyticus* in shellfish with a sensitivity of detecting these genes at levels of 1 - 10 cells/g after a pre-enrichment (8 h) of sample homogenate in APW. Presence of the *tl*, *tdh* and *trh* genes in a sample resulted in appearance of bands with molecular weights of 450, 269 and 500 bp, respectively, on agarose gel.

Other genetic markers have been reported for detecting *V. parahaemolyticus*. The *toxR* gene, which was initially described as a regulatory gene in *V. cholerae*, was found in *V. parahaemolyticus* (Lin and others 1993). Kim and others (1999) developed an assay for identification of *V. parahaemolyticus* by PCR targeting the *toxR* gene. R72H DNA sequence, which is highly conserved in *V. parahaemolyticus*, can be used as a species-specific marker (Lee and others 1995). Venkateswaran and others (1998) proposed the *gyr*B gene, which encodes the B subunit of DNA gyrase and is essential to DNA

replication, has conserved regions for development of PCR primers detecting *V. parahaemolyticus.*

PCR-based methods have been used to detect *V. parahaemolyticus* in environmental and food samples (Bej and others 1999; Lee and others 2008; Zimmerman and others 2007). Even though the methods are highly specific, they require post amplification analysis (agar rose gel electrophoresis), which increases possibility of contamination.

The real-time PCR offers a rapid and quantitative analysis by eliminating the need for post PCR processing to measure the accumulation of PCR products after amplification. Blackstone and others (2003) developed a real-time PCR to detect pathogenic *V. parahaemolyticus* in oysters by targeting the *tdh* gene in a TaqMan system. Nordstrom and others (2007) optimized a multiplex real-time PCR targeting the *tl*, *tdh* and *trh* genes of *V. parahaemolyticus* for detecting total and pathogenic *V. parahaemolyticus* in oysters. The multiplex assay detected <10 CFU/ 2µl reaction template of pathogenic *V. parahaemolyticus* in presence of >10^4 CFU/2µl reaction template of total *V. parahaemolyticus*. Takahashi and others (2005) developed a TaqMan real-time PCR targeting *toxR* for detecting total *V. parahaemolyticus* in shellfish and seawater. The investigators reported a very small difference (<10 fold) in number of this organism between culture method and the PCR method. A real-time PCR targeting *gyr*B gene was also developed for quantitative detection of *V. parahaemolyticus* in seafood (Cai and others 2006). Ward and Bej (2006) developed a real-time PCR targeting *tl*, ORF8, *tdh*, *trh* genes of *V. parahaemolyticus* in shellfish. This assay was able to detect total and pathogenic *V. parahaemolyticus* with an initial inoculum of 1 CFU/g of *V. parahaemolyticus* in oysters after 16 h of enrichment in T_1N_1 broth (1% tryptone and 1% NaCl) at 35 °C.

1.6.3 DNA hybridization

DNA hybridization using a non-radioactive probes (Alkaline phosphatase (AP)-labeled or digoxigenin (DIG)-labeled DNA probes) has been developed for detection of *V. parahaemolyticus* (BAM 2004). This technique involves (a) fixation and pretreatment

of sample, (b) synthesis of labeled probes, (c) hybridization of probe followed by washing to remove unbound probe, and (d) probe detection through development of color. McCarthy and others (1999) evaluated AP- and DIG-labeled oligonucleotide probes for detection of the *tl* gene and reported that results obtained from both probes were in 98% agreement. Gooch and others (2001) developed AP-labeled and DIG-labeled probes targeting the *tl* gene of *V. parahaemolyticus* after direct plating onto T_1N_3 (1% Tryptone, 3% NaCl and 2% agar) medium and reported comparable results between colony hybridization and MPN methods. The AP- and/or DIG-labeled DNA probes for detecting *tl* and *tdh* genes were used for enumeration of total and pathogenic *V. parahaemolyticus* in shellfish after direct plating of samples on T_1N_3 plates (Deepanjali and others 2005; DePaola and others 2000). Using AP-labeled *tl* and AP-labeled *tdh* probes detecting pathogenic *V. parahaemolyticus* in seafood samples, Raghunath and others (2008) reported that 4 out of 30 samples were positive for tdh^+ by colony hybridization following direct plating of overnight-enriched samples. On the other hand, pathogenic *V. parahaemolyticus* was detected in only one sample by traditional direct-plating colony hybridization. This finding indicates direct plating colony hybridization following enrichment increases the sensitivity for detection of pathogenic *V. parahaemolyticus* from seafood.

1.7 Control

  V. parahaemolyticus is a natural inhabitant of marine and estuarine environments. Therefore, seafood can be easily contaminated with *V. parahaemolyticus*, especially in the summer months when water temperature increases. It is known that this bacterium can multiply rapidly to an infectious level in seafood if temperature abuse occurs at some points between harvest and consumption. Although *V. parahaemolyticus* can be destroyed by adequate cooking, presence of this pathogen in shellfish, particularly oysters, intended for raw consumption is a safety concern.

  According to a dose response model, about 50 to 100% of the human subjects became ill after ingestion of pathogenic *V. parahaemolyticus* ($>1\times10^6$) (FDA 2005). The

U.S. FDA (2011) has established a limit of no more than 10,000 cells/g of total viable cells of *V. parahaemolyticus* in minimally-processed seafood for human consumption. Limiting exposure time to elevated temperature by rapid chilling upon harvest as well as providing proper information whether products are intended for raw consumption or for cooking only will help the industry to provide safe products to consumers and promote consumer awareness of product safety. In addition, a number of postharvest treatments have been developed for reducing risks of *V. parahaemolyticus* infection associated with shellfish consumption.

1.7.1 Limitation of time from harvest to refrigeration

The levels of *V. parahaemolyticus* in shellfish at harvest vary depending on temperatures of waters at harvest. A study reported that the average density (130 CFU/g) of *V. parahaemolyticus* in American oysters (*Crassostrea virginica*) in warm water (>20 °C) were higher than that (15 CFU/g) in the oysters when water temperature was lower than 20 °C (Gooch and others 2002). The numbers of *V. parahaemolyticus* in shellstock oysters harvested from the warmer waters increased quickly by 0.8, 1.7 and 2.9 log CFU/g when the oysters were held at 26 °C for 5, 10 and 24 h, respectively. Holding oysters at 35 °C for 2 to 3 days resulted in considerable increase in numbers of *V. parahaemolyticus* whereas the organism survived at 4 °C for at least 3 weeks with little or no change in numbers (Johnson and others 1973). Another study reported that numbers of *V. parahaemolyticus* in oysters remained nearly unchanged after a storage at 8 °C for 7 days (Hood and others 1983). These observations indicated that storing shellfish at low temperatures could retard growth of *V. parahaemolyticus*, but not a means to inactivate the organism.

1.7.2 Labeling for intended use

Any raw and postharvest-processed shellfish is required to have a tag bearing information, including the name of the state or foreign country where the raw shellfish was harvested, harvest date, shucking date (if applicable) and sell-by-date (NSSP 2011).

Labeling may vary but needs to be adequate for product description and intended use. This regulation helps state officials to track down shellfish in the marketplace back to the distributor and to the harvester when an outbreak occurs.

1.7.2.1 Raw consumption

Risk of *Vibrio* infection is primarily associated with consumption of raw shellfish. All shellstock, half-shell and shucked shellfish intended for raw consumption are required to include a consumer advisory. For example, "Consuming raw or undercooked meats, poultry, seafood, shellfish or eggs may increase your risk of foodborne illness, especially if you have certain medical conditions".

1.7.2.2 Cooking and post-harvest processing only

One of the *V. parahaemolyticus* control plans is that shellfish should bear "For Cooking Only" or "Post-harvest Process Only" when they are harvested from a restricted harvest area or at times *V. parahaemolyticus* infection is likely to occur. The restricted area is characterized by two or more *V. parahaemolyticus* illnesses in a 3-year period or an outbreak within five years or average water temperatures exceeding 60 °F for states bordering the Pacific Ocean and 81 °F for states bordering the Gulf of Mexico and the Atlantic Ocean (NSSP 2011). Due to possible high contamination of *V. parahaemolyticus*, foodservice operations and consumers should be aware of that those products should be cooked before serving. In addition, shellfish may undergo one of approved postharvest processes, including quick freezing with extended frozen storage, pasteurization, high pressure treatment and low dose radiation, and be labeled as "processed for added safety" or "processed to reduce *V. vulnificus* and/or *V. parahaemolyticus* to non-detectable levels". The FDA has considered requiring a postharvest process for raw Gulf oysters harvested from Alabama, Florida, Louisiana, Mississippi, or Texas from April through October as a result of *V. vulnificus* contamination. Currently, California is the only state with a restriction on the sale of raw summer Gulf Coast oysters due to fatal infection from *V. vulnificus*. California

Department of Public Health reported that the median annual number of cases dropped from 5.5 (during 1991 - 2002) to 0 (during 2003 - 2010) and the median annual number of deaths also dropped from 2.5 (during 1991 - 2002) to 0 after implementation of the 2003 regulation (Vugia and others 2013).

1.7.3 Post-harvest processes

Postharvest processes are used to reduce not only spoilage bacteria but also pathogenic organisms, resulting in extending shelf life and enhancing microbial safety. In particular, any processes that is capable of reducing the level of a target pathogen (or more) in processed shellfish to non-detectable (<30 MPN/g) and achieving a minimum 3.52 log reduction could be adopted by shellfish industry (NSSP 2011). Many methods, including rapid chilling upon harvest, freezing, low-temperature pasteurization, irradiation, depuration and high hydrostatic pressure processing (HPP) can be utilized to reduce *V. parahaemolyticus* and *V. vulnificus* contamination in shellfish.

1.7.3.1 Rapid chilling

Rapid chilling of shellfish upon harvest is critical to retard growth of vibrios. An early study by Cook and Ruple (1989) showed that storage of Eastern shellstock oysters harvested from the U.S. Gulf Coast in the summer at 50 °F (10 °C) prevented multiplication of fecal coliforms and vibrios, including *V. vulnificus*. The National Shellfish Sanitation Program (NSSP 2011) has established time to temperature matrix (Table 1.2) requiring shellfish to be cooled down to <50 °F (10 °C) (control temperature) by ice, refrigeration or other means within a certain period of time depending on the average monthly maximum air temperature at the time of harvest. Shellfish harvested when the air temperature is 50 - 60 °F should be chilled to 50 °F or lower within 24 h while less exposure times are set for shellfish harvested at warmer air temperatures (18 h for >60 °F - 80 °F; 12 h for >80 °F). In addition, temperature of shellstock oysters should also be maintained at <50 °F (10 °C) during points of transfer.

Table 1.2 Time to temperature matrix for *Vibrio parahaemolyticus* (NSSP 2011)

Action level	Average monthly maximum air temperature	Maximum exposure (h) from harvest to temperature control[a]
1	<50 °F (10 °C)	36
2	50 °F - 60 °F (10 °C - 15 °C)	24
3	>60 °F - 80 °F (15 °C - 27 °C)	18
4	>80 °F (>27 °C)	12

[a] The maximum hours that allow temperature of shellfish to reduce to <50 °F (10 °C).

Even though rapid chilling can prevent rapid multiplication of bacteria upon harvest, it does not always guarantee the safety of shellfish for raw consumption. A treatment of shellstock oysters with ice immersion for 3 h followed by refrigeration storage at 45 °F resulted in a small decrease (from 2.9 to 2.2 log CFU/g) of *V. vulnificus* in the oysters after 7 days and an increase (to 4.0 log CFU/g) after 14 days of storage (Quevedo and others 2005). Slight increases in coliform counts (from 1.3 to 4.8 MPN/100g) and heterotrophic bacteria (from 4.6 to 5.5 log CFU/g) were also reported in ice-treated oysters after 14 days of cold storage at 45°F. Another study reported that placing oysters in ice either on board or at dockside did not significantly reduce *V. vulnificus* or *V. parahaemolyticus* during subsequent cold storage when compared to non-iced oysters (Melody and others 2008). Levels of *V. vulnificus* and *V. parahaemolyticus* in raw Gulf oysters according to a market survey between 1998 and 1999 were 5 to 6 log greater than those observed in oysters subjected to postharvest processes, such as mild heat treatment, freezing and high hydrostatic pressure, collected from June through October 2004 (DePaola and others 2009). Consumption of processed oysters could greatly reduce risk of illness associated with *Vibrio* infections (FAO/WHO 2005; FDA 2005).

1.7.3.2 Freezing and frozen storage

Freezing is a common way to preserve food for extended period of time by inhibiting growth of microorganisms. Complete inactivation of *V. parahaemolyticus* was reported in oyster homogenates (10^{5-7} cfu/g) being stored at -18 and -24 °C for 15 to 28 weeks (Muntada-Garriga and others 1995). The time required for complete inactivation of bacterial cells depends on the initial load of bacteria and freezing temperatures. Holding half-shell oysters at -20 °C for 4 to 6 months reduced number of *V. vulnificus* from <1,000 cell/g to non-detectable levels (Andrews 2004). Densities of *V. vulnificus* decreased from 150,000 to 930 MPN/g in oyster meats being individually quick frozen at -30 °C for 13 min (Cook and Ruple 1992). Storing the frozen oyster meats at -20 °C reduced numbers of *V. vulnificus* to 93 and 7.5 MPN/g after 1 and 2 weeks, respectively. However, a low number (0.9 MPN/g) of this organism *V. vulnificus* was detected in the oysters after being stored for 3 months. Liu and others (2009) evaluated a freezing method through liquid nitrogen at -95.5 °C for 12 min in half-shell oysters and reported >3.52 log reductions of *V. parahaemolyticus* after subsequent frozen storage at -20 °C for 5 months.

1.7.3.3 Low-temperature pasteurization

Pasteurization is a process of heating food products to a specific temperature for a definite length of time followed by a rapid cooling to inactivate all human pathogens but not all spoilage organisms. Thus, pasteurized products such as milk, juice, and cheeses, are required to keep refrigerated through their shelf life. Cook and Ruple (1992) reported that a heat treatment of oyster meats at 50 °C for 5 and 10 min reduced numbers of naturally-occurring *V. vulnificus* from 59,000 to 220 and <0.3 MPN/g, respectively. Heating oysters under these mild conditions did not result in a noticeable cooked appearance and taste. Andrew and others (2000) studied a similar heat treatment of shellstock oysters at 50 °C for 10 min and showed that numbers of *V. vulnificus* and *V. parahaemolyticus* decreased from 10^5 MPN/g to non-detectable levels (<3 MPN/g). Aerobic plate counts in the oysters pasteurized for 10 min was 3 log MPN/g lower than

that observed in untreated oysters and did not change after 14 days of ice storage (<4 °C). The numbers of *V. vulnificus* in the pasteurized oysters also remained non-detectable during 14 days of ice storage. These findings were corresponding to a survey reporting a very low number (<3 MPN/g) of either *V. vulnificus* or *V. parahaemolyticus* in all mild-heated oysters collected from processors and retail establishments (DePaola and others 2009).

1.7.3.4 Irradiation

Irradiation has been applied to a variety of food including fresh iceberg and spinach, red meat, poultry and shellfish to enhance safety and shelf life with different allowance dose levels (FDA 2013). In April 2014, the U.S. Food and Drug Administration amended current food additive regulations to allow the safe use of ionizing radiation (up to 6.0 kiloGray) on crustaceans (e.g., crab, shrimp, lobster, crayfish, and prawns) to control foodborne pathogens and extend shelf life. It was reported that low doses (<3.0 kGy) gamma ray and even as high as 5.0 kGy X-ray of irradiation did not kill oysters or affect their sensory attributes (Jakabi and others 2003; Mahmoud and Burrage 2009). Counts of *V. vulnificus* and *V. parahaemolyticus* in oyster meats reduced by 7 and 4 log MPN/g after being treated with gamma ray at doses of 1.0 and 1.5 kGy, respectively (Andrews and others 2003). The irradiated (<1.5 kGy) oysters also had comparable sensory attributes with a longer shelf life (>15 days) than untreated oysters stored at refrigeration temperatures. Another study reported that irradiation of 0.5 and 1.0 kGy resulted in a significant reduction of *V. vulnificus* by 4 and 5 log, respectively, in live shellstock oysters while increased doses to 1.5 and 2.0 kGy reduced numbers of *V. vulnificus* by 5 and 6 log, respectively (Kilgen and others 2001). A greater than 6 log CFU/g reduction of *V. parahaemolyticus* was observed in half shell and whole shell oysters being irradiated with 2.0 and 5.0 kGy X-Ray, respectively (Mahmoud and Burrage 2009). In addition, a treatment of 1.0 kGy X-Ray reduced an initial level of microflora in whole shell oysters from 4.7 to <1.0 log CFU/g. A similar reduction (6 log CFU/g) of *V. parahaemolyticus* was reported in artificially-inoculated oysters subjected

to gamma ray radiation at 1.0 kGy (Jakabi and others 2003). Irradiation is considered one of the most efficient processes for the decontamination of microorganisms in food. However, it has not been widely used due to the reluctance of consumers to accept irradiated food. In addition, handling radioactive materials is a safety concern which also limits its application.

1.7.3.5 Depuration

Depuration is a controlled process that allows shellfish to release contaminants from the digestive tract in clean seawater, which can be operated as a flow-through or recirculating system. The flow-through system requires continuous supply of clean seawater while the recirculating system utilizes a fixed volume of seawater which is constantly filtered or sterilized by ultraviolet light, chlorine, chlorine-containing compounds, ozone or other means. Natural seawater for shellfish depuration should be taken from areas free of chemicals or biotoxins with low levels of microorganisms. Artificial seawater can be used as an alternative if pollution-free natural seawater is not readily available. Depuration is approved in European countries as an postharvest means for decontaminating *Salmonella*, *Escherichia coli* and coliforms in live shellfish (European Communities 1991). Purified shellfish must contain less than 300 coliforms or 230 *E. coli* per 100 g of flesh and fluid with no *Salmonella* being detectable in 25 g of flesh.

Many studies have shown that vibrios appear to be more persistent in shellfish than certain bacteria, such as *Salmonella* spp., *E. coli* and coliforms during depuration process at ambient temperature (Croci and others 2002; Greenberg and others 1982). In addition, depuration at an elevated temperature may allow bacteria to multiply in water and shellfish. Kaspar and Tamplin (1993) reported that the numbers of *V. vulnificus* cells in seawater (salinity: 10 ppt) increased after incubation at temperatures ranging from 13 to 30 ºC for 2 days. Holding naturally-contaminated oysters containing 10 MPN/g of *V. vulnificus* in seawater at 23 ºC for 2 days resulted in an increase of *V. vulnificus* to 10^5 MPN/g while no change in the levels of *V. vulnificus* in the oysters was observed when

held at 15 ºC for 7 days (Tamplin and Capers 1992). On the other hand, the numbers of *V. parahaemolyticus* reduced in artificially-contaminated clams held in seawater for 3 days at 15 and 25ºC with slightly greater reductions being observed at 15 ºC (Greenberg and others 1982).

Several studies have indicated that the optimal temperature for shellfish depuration varies depending on species and bacteria to be reduced. Chae and others (2009) reported 2.88- and 2.09-log reductions of *V. vulnificus* and *V. parahaemolyticus*, respectively, in the American oysters (*Crassostrea virginica*) held in seawater at 15 ºC for 2 days. Refrigerated (5 °C) seawater depuration for 4 and 6 days resulted in greater than 3-log reductions in Pacific oysters (*Crassostrea gigas*) harvested in the winter and the summer, respectively (Su and others 2010). The process caused no significant fatality of oysters and increased the survival of the depurated oysters during subsequent cold storage at 5 °C. Arcisz and Kelly (1955) found that soft clams (*Mya arenaria*) depurated at low water temperature (2.5 ºC) was as effectively as at higher temperatures (up to 20 ºC) for reducing *E. coli* and *Salmonella schottmuelleri*. Another study investigating depuration for elimination of *E. coli* and *Salmonella charity* from the Sydney rock oysters (*Crassostrea commercialis*) reported that the process was more effective when the oysters were depurated at 18-22 ºC than at higher (24-27 ºC) or lower temperatures (13-17 ºC) (Rowse and Fleet 1984).

Salinity is another factor that can affect water-filtering activity of shellfish (Phuvasate and Su 2013). The salinity of seawater changes year round and may range from 4 to 37 ppt among coasts (Motes and others 1998). This means that shellfish may need to adjust biological activities according to the changes of salinity of the growing environments. Blake and others (1985) studied effects of salinity (15, 20, 25 and 35 ppt) of seawater on the efficacy of depuration for removing *E. coli* in the American oysters and observed 95, 98 and 90% reductions of *E. coli* in the oysters when they were depurated for 48 h in seawater with a salinity of 20, 25 and 35 ppt, respectively. However, no reduction of *E. coli* was reported when the oysters were depurated in seawater with a salinity of 15 ppt. Rowse and Fleet (1984) reported that the populations

of *E. coli* in the Sydney rock oysters were reduced by 1-2 log after 48 h of depuration in water with salinities of 16-20 ppt. However, a 3-log reduction in the populations was observed when the oysters were depurated in water with higher salinities of 43-47 ppt. Kaspar and Tamplin (1993) reported that *V. vulnificus* survived in water with salinities ranging from 5 to 25 ppt for 6 days but was completely eliminated in deionized water. The study also observed decreases in the numbers of *V. vulnificus* by 60 and 80% in seawater containing 30 and 35-38 ppt, respectively. The authors suggested that depuration with seawater with a salinity of 25 ppt or higher might result in a better decontamination of *V. vulnificus* in shellfish.

1.7.3.6 High hydrostatic pressure processing (HPP)

High hydrostatic pressure processing (HPP) is a non-thermal technology that can be used for food preservation by eliminating spoilage organisms and pathogenic bacteria, thereby improving safety and extending shelf life of food products (San Martín and others 2002). HPP has potential to produce food with higher quality and nutritional values than thermally-processed products since it does not affect covalent bonds, resulting in retaining flavor, color and nutritional components (Hayashi and others 1989; Singh and Ramaswamy 2013). Applications of HPP in many food products such as juices, guacamole, ready-to-eat meats, and shellfish have been reported (Bajovic and others 2012; Bull and others 2004; Considine and others 2008; Murchie and others 2005).

Inactivation of microorganisms by HPP may be associated with a number of changes in cell membranes, cell morphology and biochemical reactions (Hoover and others 1989; Smelt 1998). Cell membranes play an important role and are the first point of attack under pressures, resulting in phase transition of membrane lipids and alterations in membrane permeability (Chong and Cossius 1983; Mañas and Pagán 2005). A direct relationship between membrane integrity and viability has been reported in yeasts and bacteria exposed to high pressures (Pagán and Mackey 2000; Shimada and others 1993; Tholozan and others 2000). Other reports showed changes in cell morphology observed

with electron microscopy (Ritz and others 2001; Ritz and others 2002; Kaletunç and others 2004) and membrane-bound enzyme activity (Wouters and others 1998).

Several studies have reported resistance to high pressures varies among species and strains of foodborne pathogens (Alpas and others 1999; Cook 2003; Patterson and others 1995; Styles and others 1991). It is known that Gram-positive bacteria are more pressure-resistant than Gram-negative bacteria and bacterial spores are more pressure-resistant than vegetative cells (Arroyo and others 1997). In addition, pressure resistance is dependent on physiological state of the organisms with exponential-phase cells being more sensitive to pressure than stationary-phase cells (Pagán and Mackey 2000).

Many studies have investigated effects of HPP at various pressures, temperatures and time combinations on reducing microbial loads and pathogenic vibrios in oysters (Kural and Chen 2008; Kural and others 2008; Linton and others 2003; Prapaiwong and others 2009). A treatment of 241 MPa for 2 min at 27-29 °C was capable of reducing *V. vulnificus* in shellstock oysters and homogenized oyster meats by > 4.8 and >5.0 log units, respectively, whereas a higher pressure treatment of 300 MPa for 2 min at 24-25 °C resulted in only 4.0-log reduction of *V. parahaemolyticus* O3:K6 in shellstock oysters (Cook 2003). A 2-min treatment of oyster meats at 250 and 300 MPa resulted in significantly greater reductions of *V. parahaemolyticus* at 1 °C (3.1 and 4.9 log) than at 20 °C (2.1 and 3.9 log) (Kural and others 2008). A 4-min treatment of 150 MPa at -2 °C reduced counts of *V. vulnificus* by 4.7 log while a smaller reduction (0.5 log) of this organism were observed at 20-30 °C (Kural and Chen 2008). These findings indicate that pronounced inactivation of microorganisms could be achieved at a pressure treatment at low temperatures. A validation process studied by Ma and Su (2011) reported >3.52-log reductions of *V. parahaemolyticus* in shellstock oysters after a treatment of 293 MPa for 2 min with water temperature of 8±1 °C. The pressure-processed oysters had a shelf life of 6-8 or 16-18 days when stored at 5 °C or in ice, respectively. HPP is an effective means to inactivate pathogenic vibrios in oysters and also maintain raw-like quality. An added benefit from the process is that oysters shuck under the pressure (Calik and others 2002; Cruz-Romero and others 2004; He and others 2002; Ye and others 2012).

1.8 Conclusion

     V. parahaemolyticus is an important seafood-borne pathogen for public health concerns and a major cause of illness associated with consumption of seafood, particularly shellfish, worldwide. Many studies have investigated effects of environmental factors on the prevalence of the bacterium in water and shellfish at various regions. Two hemolysins (TDH and TRH) have been identified as virulence factors in most clinical strains, but the infectious doses of virulent *V. parahaemolyticus* remain unclear. Understanding the risk posed by this organism and development of effective post-harvest processes will significantly minimize the risks of *V. parahaemolyticus* infection associated with seafood consumption. The objectives of this research were to develop low-temperature depuration and low-temperature high hydrostatic pressure processing (HPP) for reducing *V. parahaemolyticus* in oysters. Understanding factors affecting the efficacy of depuration and high pressure processing as well as pressure resistance of *V. parahaemolyticus* and mechanism of *V. parahaemolyticus* inactivation by pressure will facilitate post-harvest treatments to successfully eliminate the pathogen.

CHAPTER 2

REDUCTION OF *Vibrio parahaemolyticus* IN PACIFIC OYSTERS (*Crassostrea gigas*) BY DEPURATION AT VARIOUS TEMPERATURES

Sureerat Phuvasate, Ming-Hui Chen and Yi-Cheng Su

2.1 Abstract

Consumption of raw oysters has been linked to several outbreaks of *Vibrio parahaemolyticus* infection in the United States. This study investigated effects of ice storage and UV-sterilized seawater depuration at various temperatures on reducing *V. parahaemolyticus* in oysters. Raw Pacific oysters (*Crassostrea gigas*) were inoculated with a mixed culture of five clinical strains of *V. parahaemolyticus* (10290, 10292, 10293, BE 98-2029 and 027-1c1) at levels of 10^{4-6} MPN/g. Inoculated oysters were either stored in ice or depurated in recirculating artificial seawater at 2, 3, 7, 10, 12.5, and 15 ºC for 4 to 6 days. Holding oysters in ice or depuration of oysters in recirculating seawater at 2 or 3 °C for 4 days did not result in significant reductions ($P > 0.05$) of *V. parahaemolyticus* in the oysters. However, depuration at temperatures between 7 and 15 ºC reduced *V. parahaemolyticus* populations in oysters by >3.0 log MPN/g after 5 days with no loss of oysters. Depuration at refrigerated temperatures (7-15 ºC) can be applied as a post-harvest treatment for reducing *V. parahaemolyticus* in Pacific oysters.

Keywords: *Vibrio parahaemolyticus*, Pacific oyster, Depuration, Shellfish, Seafood safety

2.2 Introduction

   Vibrio parahaemolyticus is a foodborne pathogen that occurs naturally in the marine environments (Huq and Colwell 1995) and frequently isolated from seafood throughout the world (Costa Sobrinho and others 2011; Hervio-Heath and others 2002; Sujeewa and others 2009; Costa Sobrinho and others 2010). This pathogen is recognized as the leading cause of gastroenteritis associated with seafood consumption in the United States (Daniels and others 2000a; Su and Liu 2007). It is estimated that 4,500 cases of *V. parahaemolyticus* infections occur each year in the U.S. with 62% of them (2,800 cases) being associated with consumption of raw oysters (FDA 2005). A recent *V. parahaemolyticus* outbreak in 2006 affecting 177 residents of three states, New York, Oregon and Washington, was traced to oysters harvested from Washington and British Columbia, Canada and sold nationwide (CDC 2006).

   The occurrence of *V. parahaemolyticus* in the environments and oysters is known to correlate to water temperature with the bacterium often being detected during summer months when the water temperature exceeds 15 ºC (Kaneko and Colwell 1973; Kelly and Stroh 1988; Ristori and others 2007; Costa Sobrinho and others 2010). To reduce the risks of infections caused by *Vibrio* spp. associated with shellfish consumption, the U.S. National Shellfish Sanitation Program (NSSP 2011) has established time/temperature regulations that limit maximum hours of holding shellfish from harvest to refrigeration (≤10 ºC) depending on average maximum air temperature upon harvest. Hood and others (1983) observed that levels of naturally contaminated *V. parahaemolyticus* in shellstock oysters decreased slightly when the oysters were stored at 2 and 8 ºC. A small reduction (0.39 log MPN/g) of naturally occurring *Vibrio vulnificus* was reported in shellstock oysters after being held at 4 ºC for 14 days (Cook and Ruple 1992). Although growth of *Vibrio* spp. in shellfish can be inhibited at refrigeration temperatures, the bacteria can multiply rapidly in shellfish once exposed to elevated temperatures (>25 ºC). Studies have shown that populations of *V. parahaemolyticus* in unrefrigerated oysters increased rapidly to 50 to 790 folds of its original level within 24 h of harvest when oysters were exposed to an elevated temperature (Gooch and others 2002). Therefore, keeping oysters

in cold chain alone may not be sufficient to eliminate risks of *Vibrio* infections associated with raw oyster consumption.

Oysters are filter-feeding animals, which can filter large volumes of water (up to 13 L/h) for nutrients (Loosanoff 1958), and can accumulate microorganisms including human pathogens present in the growing environments. Many attempts have been made to develop processing technologies to reduce number of bacteria including *Vibrio* spp. in oysters for safe consumption. Post-harvest processes, including low-temperature pasteurization (Andrews and others 2000), flash freezing followed by frozen storage (Liu and others 2009), high-pressure processing (Kural and Chen 2008; Kural and others 2008; Ma and Su 2011) and irradiation (Andrews and others 2003), have been developed for inactivating *V. parahaemolyticus* in oysters. However, these processes either require high initial investment or operation costs and oysters are often killed during the processes, except by low-dose irradiation. A simple and cost-effective process for reducing *V. parahaemolyticus* in oysters without significant adverse effects remains to be developed.

Depuration is a controlled purification process by holding shellfish in seawater disinfected by ultraviolet (UV) light, chlorine or ozone (Blogoslawski and Stewart 1983). The process at ambient temperatures had been reported effective in reducing *Salmonella*, *Esherichia coli* and coliforms but not for eliminating *Vibrio* spp. in shellfish (Eyles and Davey 1984; Son and Fleet 1980; Timoney and Abston 1984; Vasconcelos and Lee 1972). However, decreasing temperature for depuration to 15 ºC has been reported capable of reducing *V. parahaemolyticus* and *V. vulnificus* in the Gulf oysters (*Crassostrea virginica*) by 2.1 and 2.9 log MPN/g, respectively, after 48 h (Chae and others 2009). Recently, we investigated depuration with refrigerated seawater at 5˚C for reducing *V. parahaemolyticus* in the Pacific oysters and reported that the process reduced *V. parahaemolyticus* populations in Pacific oysters by >3.0 log MPN/g after 96-144 h (Su and others 2010). These results suggest that the efficacy of depuration for reducing *V. parahaemolyticus* in oysters depends on the temperature of operation. The objective of this study was to study temperature effects on depuration for reducing *V.*

parahaemolyticus in the Pacific oysters and identify an optimal depuration for post-harvest processing of oysters without any adverse effects.

2.3 Material and methods

2.3.1 Bacterial culture preparation

Five clinical strains of *Vibrio parahaemolyticus* (10290, 10292, 10293, BE98-2029 and 027-1c1) obtained from the culture collection of the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, WA) were used in this study. Each strain was grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Spark, MD) containing 1.5% NaCl (TSB-Salt) at 37 ℃ for 18-24 h. The enriched culture was streaked onto individual plates of tryptic soy agar (TSA; Difco, Becton Dickinson) supplemented with 1.5% NaCl (TSA-Salt) and incubated at 37 ℃ for 18-24 h. A single colony formed on a TSA-Salt plate was transferred to TSB-Salt and incubated at 37 ℃ for 4 h. Enriched cultures of *V. parahaemolyticus* were pooled into a 50-mL sterile centrifuge tube and harvested by centrifugation at 3000 x g (Sorvall RC-5B, Kendro Laboratory Products, Newtown, CT) at 5 ℃ for 15 min. Pellet cells were re-suspended in 2% salt solution to produce a culture suspension of approximately 10^{8-9} CFU/ml.

2.3.2 Oyster preparation

Raw Pacific oysters (*Crassostrea gigas*, diploid, 7.8 ± 0.7 cm long and 12-18 months old) were obtained from an oyster farm in the Yaquina Bay in Newport of Oregon between March and May (water temperature ranged from 9 to 14 ℃) and between September and January (water temperature ranged from 7 to 15 ℃) to avoid using oysters containing naturally accumulated *V. parahaemolyticus* in the studies. The oysters were briefly washed with tap water and placed in a tank (45 by 30 by 30 cm; Nalgene, Rochester, NY) containing aerated artificial seawater (ASW) with a salinity of 30 parts per thousand (ppt). The ASW was prepared by dissolving Instant Ocean Salt (Aquarium systems, Inc., Mentor, OH) in deionized water according to the manufacturer's instruction. After being held in ASW at room temperature for 2-4 h,

oysters were used for movement study, analysis of naturally-accumulated *V. parahaemolyticus* and inoculation with *V. parahaemolyticus*.

2.3.3 Determination of oyster movement

Oyster movement in ASW (15 L) at various temperatures was studied using a Gape Ometer (Pacific Shellfish Institute, Olympia, WA) with eight oysters each time. The Gape Ometer consists of four rectangular bars each containing an electronic device capable of measuring the distance between the surface of the bar and a magnetic sensor. For determination of oyster movement, two oysters were glued to each bar with a magnetic sensor attached to the upper shell of each oyster. The oysters were placed in a polystyrene foam cooler containing ASW being circulated using a pump (Mini-jet 606, Aquarium systems, Italy) at 320 L/h through a water chiller (EU-CL85, AquaEuroUSA, Gardena, CA*)* at 3, 7, 10, 15 or 20 ºC. The distance between the rectangular bar and magnetic sensor was recorded every 5 minutes for 24 h in a computer. Changes in the distance (>0.05 cm) indicated the movement of oysters.

2.3.4 Inoculation of oysters with *V. parahaemolyticus*

For each experiment, 45 oysters were exposed to *V. parahaemolyticus* cocktail at a level of $10^{4\text{-}5}$ CFU/ml in freshly prepared ASW (20 L). Accumulation of *V. parahaemolyticus* in oysters was conducted according to previously published procedures in aerated ASW at room temperature overnight (16-18 h) with water being circulated (15 L/h) (Su and others 2010).

2.3.5 Ice treatment and low temperature depuration

Oysters inoculated with *V. parahaemolyticus* were either stored with ice covered in a chest cooler for 4 days or depurated with ASW at various temperatures (2, 3, 7, 10, 12.5 and 15 ºC) for 4 to 6 days. For depuration study, the oysters were held in 60 L of ASW in a laboratory-scale recirculating (1500 L/h) system equipped with a 15 W Gamma UV sterilizer (Current-USA Inc., Vista, CA), a water chiller (Delta Star, Aqua

Logic, Inc., San Diego, CA) and a temperature regulator capable of controlling water temperature between 2 and 15 ºC with an accuracy of ± 0.5 ºC. The depuration was conducted at 2 and 3 ºC for 4 days and at 7, 10, 12.5 and 15 ºC for 6 days. Survival of oysters during depuration was monitored daily. Oysters which opened shells during the process and did not close upon touch were considered dead and discarded.

2.3.6 Microbiological analysis

Concentrations of *V. parahaemolyticus* in oysters before and after inoculation as well as during ice storage or depuration were determined using the three-tube most-probable-number (MPN) methods (BAM 2004). Five oysters were randomly picked for analysis at each test time. Each oyster was shucked with a sterile knife and shucked oyster meat was homogenized with an equal volume of sterile alkaline phosphate buffer saline (PBS; pH 7.4) at high speed for 1 min using a two-speed laboratory blender (Waring Laboratory, Torrington, CT). Twenty-five grams of oyster homogenate sample (1:2 dilution) was mixed with 100 ml of PBS to prepare 1:10 dilution sample suspension. Additional 10-fold dilutions of the sample suspension were prepared using PBS. All sample dilutions were individually inoculated into 3 tubes of alkaline peptone water (APW). Inoculated APW tubes were incubated at 37 ºC for 16-18 h. A loopful (3-mm inoculating loop) of each enriched APW from positive (turbid) tubes was streaked onto thiosulfate-citrate bile salt-sucrose (TCBS; Difco, Becton Dickinson) plates and incubated at 37 ºC for 18-24 h. Formation of colonies that were round and green or bluish on the plates were considered positive for *V. parahaemolyticus*. Concentrations of *V. parahaemolyticus* were determined using 3 tube MPN table by converting the number of APW tubes that were positive for *V. parahaemolyticus*. Results were reported as the mean of five determinations. The efficacy of the UV sterilizer in inactivating *V. parahaemolyticus* cells released from oysters into the recirculating water was analyzed for *V. parahaemolyticus* daily by plating water samples on TCBS plates followed by incubation at 37 ºC for 24 h.

2.3.7 Statistical analysis

Results of microbiological tests were converted to $\log_{10}$ values before being analyzed with ANOVA and Tukey's test using SPSS 13.0 software (Chicago, IL, USA). Significant differences among means of each treatment over time were established at a level of $P < 0.05$. Reductions of *V. parahaemolyticus* in oysters over time during depuration were estimated by linear regression with coefficient of determination (R^2).

2.4 Results and discussion

The movement of oysters in seawater at temperatures between 3 and 20 ºC is reported in Table 2.1. Oyster movement was rarely detected when oysters were held at 3 ºC but the movement was more frequently observed when oysters were exposed to temperatures at 7 ºC or higher. Most gape distances recorded for oyster movement were smaller than 0.50 cm. However, oyster gape could be as big as 1.25 cm. Although it is not clear whether shell movement measured by gap distances is associated with the water pumping activity of oysters, this study demonstrated that oysters were able to acclimate to environments with temperatures as low as 7 ºC and exhibited water-pumping activity (Figure 2.1).

It has been reported that the Gulf oysters (*Crassostrea virginica*) could pump water at rates of 4 and 7 L/h at 15 and 20 ºC, respectively, but the rates gradually decreased to <1 L/h when water temperature decreased to 10 ºC (Loosanoff 1958). In this study, we observed that only a few oysters (25%) showed minimal water-pumping activity when water temperature dropped to 3 ºC (Table 2.1). While the water-pumping activity is reduced when exposed to low temperature (<10 ºC), oysters were capable of acclimating to new environments and slowly resume water-pumping activity to a degree similar to that observed at 20 ºC (Figure 2.1). However, the time required for oysters to resume water-pumping activity upon exposure to low-temperature environments may vary among oysters. In this study, the water temperatures at the times of harvesting oysters ranged from 7 to 15 °C. It is believed that oysters were able to acclimate quickly

Table 2.1 Movement of oysters in artificial seawater at various temperatures for 24 h.

Temperature (°C)	Total number of oysters	Number of oysters with the maximum gape (cm) range					
		No movement[a]	0.06 to 0.25	>0.25 to 0.50	>0.50 to 0.75	>0.75 to 1.00	>1.00
3	8	6	2	-	-	-	-
7	8	3	5	-	-	-	-
10	8	1	2	2	1	2	-
15	8	2	3	1	-	1	1
20	8	1	4	3	-	-	-

[a] Recorded gape distances were not greater than 0.05 cm.
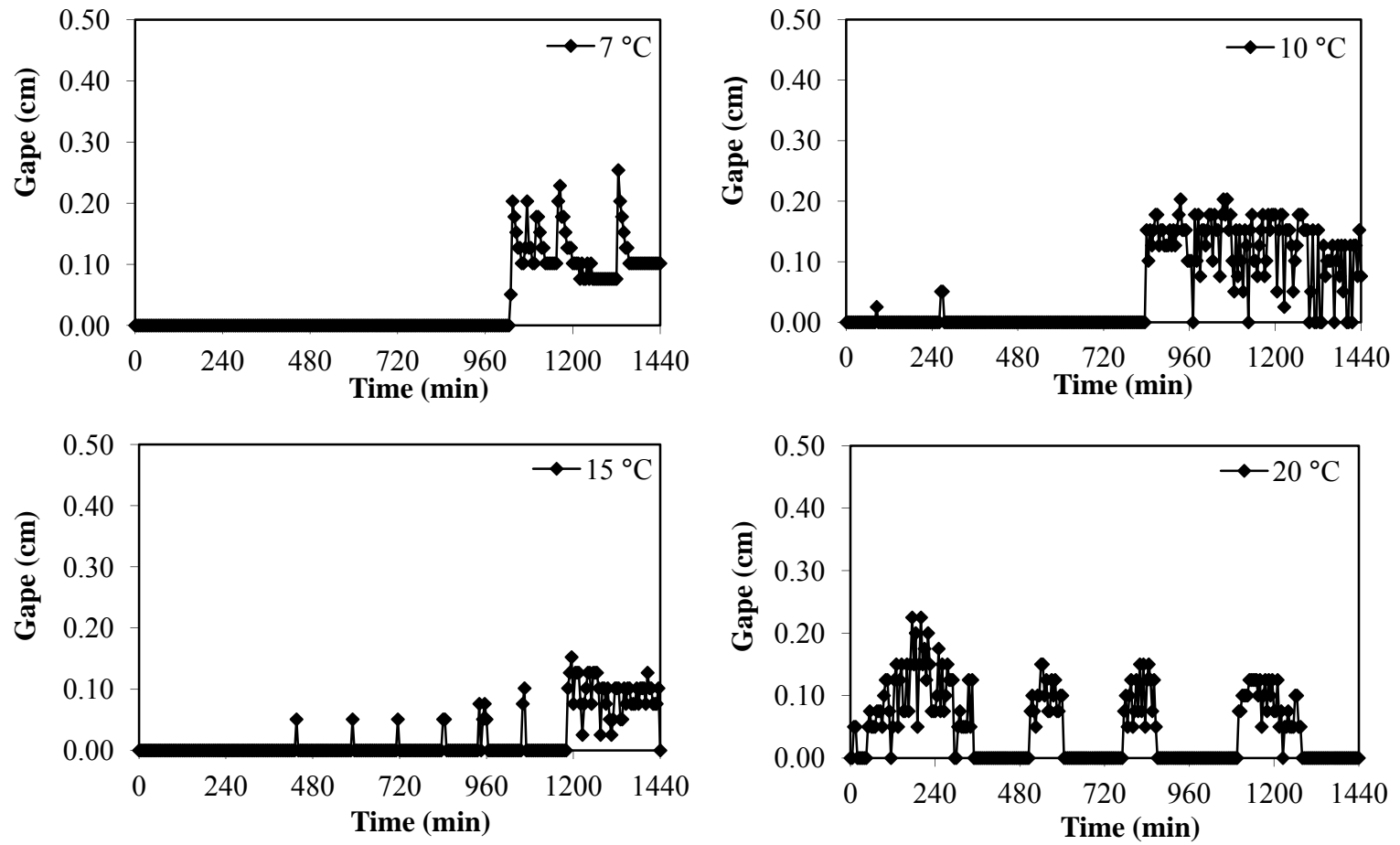
Figure 2.1 Oyster movement in artificial seawater at various temperatures

to the depuration temperatures (7-15°C) similar to those of the growing environments and resume normal water-pumping activity. Although no experiment was conducted with oysters harvested in the summer months when water temperature may increase to around 20 °C (Duan and Su, 2005), it is hypothesized that oysters would acclimate quickly to a depuration temperature of 12.5 or 15 °C. Such a hypothesis will need to be verified by conducting depuration of oysters with naturally accumulated *V. parahaemolyticus* in a summer month.

There was no naturally accumulated *V. parahaemolyticus* detected (<3 MPN/g) in oysters used in this study. This is probably because the seawater temperature of the Yaquina Bay was not higher than 15 °C when oysters were harvested. Duan and Su (2005) previously reported low levels of *V. parahaemolyticus* in oysters harvested from the Yaquina Bay (3.6-43 MPN/g) during summer months (June – August). This was in accordance with a survey conducted in the U.S. reporting geometric mean densities of *V. parahaemolyticus* in retail Pacific oysters were usually low (<3 MPN/g) year round except in the summer (39 MPN/g) (Cook and others 2002b). However, a recent survey reported that densities of *V. parahaemolyticus* in retail oysters harvested from the Pacific regions varied greatly and ranged from non-detectable (50%) to greater than 4 log MPN/g (1.7%) (DePaola and others 2010). High levels (>10,000 cells/g) of *V. parahaemolyticus* have also been reported in retail oysters harvested from the Mid-Atlantic and Gulf regions of the U.S. (DePaola and others 2010), in china (Chen and others 2010) and in Brazil (Costa Sobrinho and others 2011). Presence of high levels of *V. parahaemolyticus* in retail oysters is a health concern because densities of *V. parahaemolyticus* could increase rapidly by 1.7 and 2.9 log CFU/g in oysters after being exposed to 26 ºC for 10 and 24 h, respectively (Gooch and others 2002). Therefore, keeping oysters at low temperatures after harvest is critical in preventing rapid growth of *V. parahaemolyticus* in oysters before consumption.

However, this study found that storing whole oysters covered with ice had little effects on the reduction of *V. parahaemolyticus* in oysters. The densities of *V. parahaemolyticus* in whole oysters decreased slightly (<0.8 log MPN/g) during four days

Table 2.2 Changes of *Vibrio parahaemolyticus* populations (Log$_{10}$ MPN/g) in laboratory-contaminated oysters during ice storage and depuration at 2 and 3 ºC.

Time (day)	Ice storage [a]	Depuration temperature (ºC) [b]	
		2	3
0	4.72 ± 0.25[c] A	5.62 ± 0.74 A	5.50 ± 0.69 A
1	4.27 ± 0.74 (0.45)[d] A	5.49 ± 0.15 (0.13) A	5.06 ± 0.46 (0.45) AB
2	4.04 ± 0.45 (0.68) A	5.29 ± 0.99 (0.33) A	3.58 ± 0.94 (1.92) AB
3	3.97 ± 0.54 (0.75) A	5.47 ± 1.13 (0.15) A	3.51 ± 1.08 (1.99) B
4	4.15 ± 0.73 (0.57) A	4.91 ± 1.07 (0.71) A	4.19 ± 1.57 (1.31) AB

[a] Study was conducted in May. [b] Studies were conducted in March. [c] Values were reported as means of five oyster samples ± standard deviation. Data with the same letter in the same column were not significantly different ($P > 0.05$). [d] Reduction (log$_{10}$ MPN/g) of *V. parahaemolyticus* after treatments.

of storage in ice, but the reductions were not significant ($P > 0.05$) (Table 2.2). This is similar to previous reports of observing a small reduction (about 0.73 log MPN/g) of *V. vulnificus* in shellstock Gulf oysters stored in ice for 7 days (Cook and Ruple 1992) and about 1 log MPN/g reduction of *V. vulnificus* in Pacific oysters after 7 days of storage at 0.5 ºC (Kaysner and others 1989). These findings indicate that *V. parahaemolyticus* and *V. vulnificus* can survive in whole oysters stored in ice or at near freezing temperature, and storing oysters in ice is not a means to inactivate these pathogens.

Depuration of oysters in ASW at 2 or 3 ºC did not result in significant reductions of *V. parahaemolyticus* in oysters, though slightly greater reductions were observed at 3 than at 2 ºC (Table 2.2). The limited reductions of *V. parahaemolyticus* in oysters during storage in ice or depuration at near freezing temperatures were probably related to minimal biological activity of oysters at such low temperatures. The investigation of oyster gaping at 3 ºC indicated that the majority (75%) of oysters did not show shell movement for 24 h (Table 2.1). The lack of biological activity plus physiological variability among oysters might be the factors contributing to inconsistent reductions of *V. parahaemolyticus* in oysters observed under such processes.

Increasing temperature of depuration to 7 ºC and higher significantly increased reductions of *V. parahaemolyticus* in oysters. Densities of *V. parahaemolyticus* in the laboratory-inoculated oysters were significantly ($P < 0.05$) reduced by 1.9-2.0 log MPN/g after one day of depuration at temperatures between 7 and 15 ºC (Table 2.3). The reductions increased to 2.8-2.9 log MPN/g in oysters after four days of processes. All processes were able to yield >3.0 log MPN/g reductions of *V. parahaemolyticus* in oysters after five days. Analysis of water samples collected during the depuration processes did not find viable cells of *V. parahaemolyticus* in water (<1 CFU/10 mL), indicating the UV sterilizer worked functionally to inactivate *V. parahaemolyticus* cells released from oysters into the water.

Many studies have indicated that depuration at ambient water temperatures had little effects on reducing *Vibrio* spp. Ren and Su (2006) found no apparent change in

Table 2.3 Changes of *Vibrio parahaemolyticus* populations (Log_{10} MPN/g) in laboratory-contaminated oysters during depuration at 7, 10, 12.5, and 15 ºC.

Time (day)	Temperature [a] (ºC)			
	7	10	12.5	15
0	5.91 ± 0.30[b]A	6.30 ± 0.18 A	4.83 ± 0.69 A	6.30 ± 0.18 A
1	4.04 ± 0.44 B (1.87)[c]	4.30 ± 0.42 B (2.00)	2.96 ± 0.33 B (1.87)	4.36 ± 0.29 B (1.93)
2	3.68 ± 0.33 BC (2.23)	3.95 ± 0.45 BC (2.35)	2.58 ± 0.75 BC (2.27)	3.87 ± 0.38 BC (2.43)
3	3.31 ± 0.42 BC (2.60)	3.78 ± 0.27 BC (2.51)	2.44 ± 0.13 BC (2.39)	3.80 ± 0.39 BC (2.50)
4	3.07 ± 0.66 CD (2.84)	3.37 ± 0.25 CD (2.93)	1.86 ± 0.39 BC (2.96)	3.40 ± 0.27 CD (2.90)
5	2.63 ± 0.43 D (3.28)	3.25 ± 0.44 CD (3.05)	1.50 ± 0.83 BC (3.33)	2.98 ± 0.38 D (3.32)
6	2.50 ± 0.52 D (3.40)	3.01 ± 0.51 D (3.29)	1.39 ± 0.62 C (3.43)	3.04 ± 0.14 D (3.26)

[a] Studies were conducted in September (7 ºC), January (10 ºC) and December (12.5 and 15 ºC). [b] Values were reported as means of five oyster samples ± standard deviation. Data with the same letter in the same column were not significantly different ($P > 0.05$). [c] Reduction (Log_{10} MPN/g) of *V. parahaemolyticus* after treatments.

levels of *V. parahaemolyticus* or *V. vulnificus* in artificially contaminated oysters depurated in ASW (salinity; 29.6 ppt) at ambient temperature for up to 24 h. Limited reductions of *V. parahaemolyticus* (1.2 log MPN/g) and *V. vulnificus* (2.0 log MPN/g) were observed in the Gulf oysters after depuration with a UV sterilizer at 22 ºC for 48 h (Chae and others 2009). Tamplin and Capers (1992) reported that levels of *V. vulnificus* accumulated naturally in Gulf oysters increased by 5 log MPN/g after depuration in UV-sterilized water at 23 ºC for the same period of time. The ineffectiveness of depuration at ambient temperatures for reducing levels of *V. parahaemolyticus* or *V. vulnificus* in oysters might be due to multiplication of *Vibrio* cells in oyster tissues at warm temperatures. Our previous study reported that depuration with refrigerated seawater at 5 ºC for 96-144 h reduced *V. parahaemolyticus* populations by >3.0 log MPN/g in the Pacific oysters without significant fatality of the oysters (Su and others 2010). This study showed that depuration at 7-15 ºC for five days could also achieve the same degree of reduction (>3.0 log MPN/g) of *V. parahaemolyticus* in oysters with no mortality (data not shown).

Linear estimates of reductions of *V. parahaemolyticus* in oysters during depuration at various temperatures (7-15 ºC) indicated similar R^2 values (0.60-0.61) for depurations at 7 and 10 ºC, which were slightly lower than those observed from depurations at 12.5 (0.66) and 15 ºC (0.70) (Figure 2.2). This might be due to variability in the biological activity of oysters at lower water temperatures (7 and 10 ºC), resulting in a slight difference in bacterial reductions throughout a depuration process. The reduction models suggested that the processes of oysters at 7, 10, 12.5 and 15 ºC could achieve a 3.52-log reduction of *V. parahaemolyticus*, a guideline for post harvest processing of shellfish established by the National Shellfish Sanitation Program (NSSP 2011), within 148, 164, 137 and 155 h, respectively. The longer times (148-164 h) estimated for depuration processes at 7, 10 and 15 ºC than 137 h at 12.5 ºC could be due to higher contamination levels (5.9-6.3 log MPN/g than 4.8 log MPN/g) of *V. parahaemolyticus* in oysters It has been reported that efficacy of depuration could be influenced by the loads of pathogens in oysters. A depuration at 16-18 ºC for 36 h reduced *Salmonella* spp. in
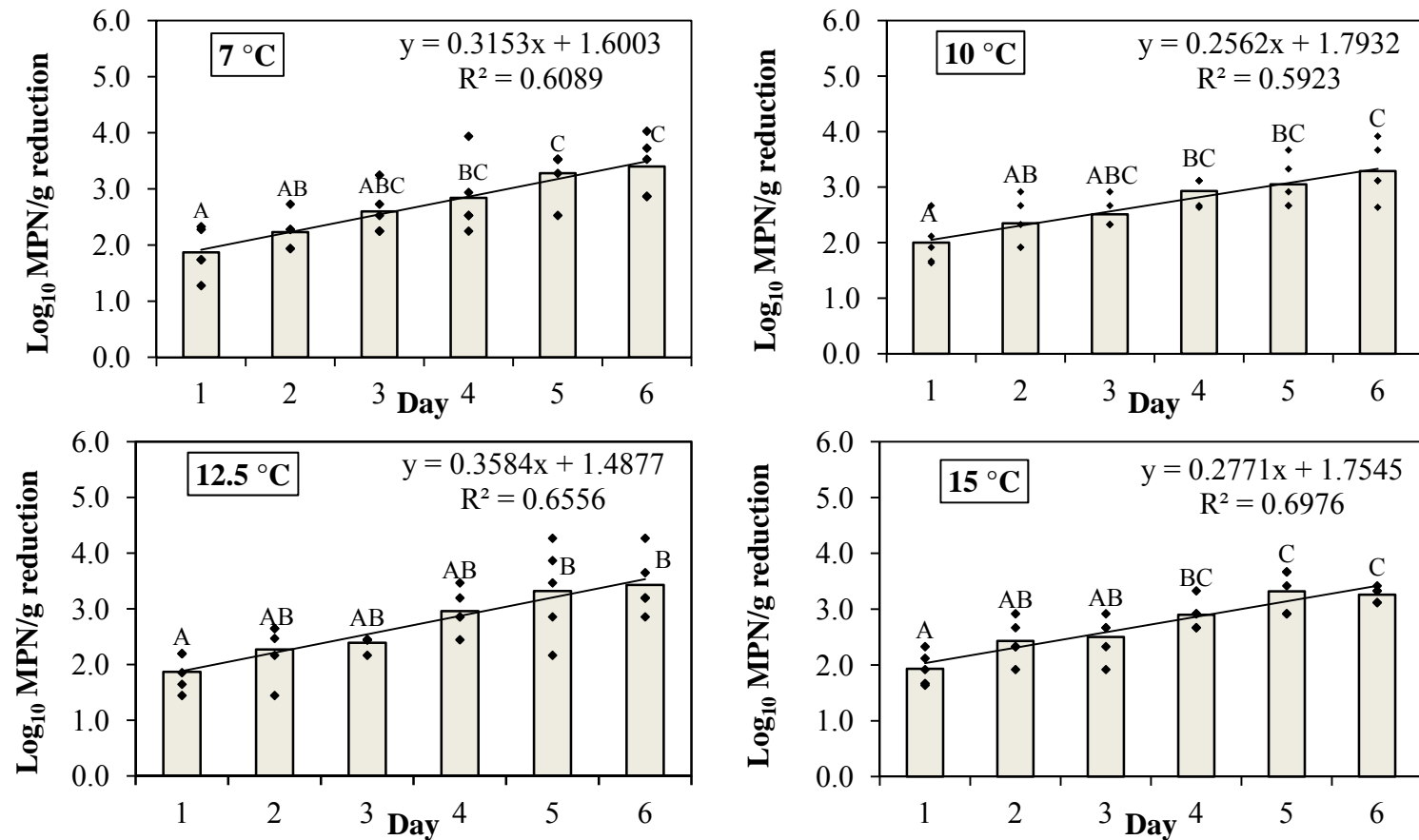
Figure 2.2 Reductions (Log_{10} MPN/g) of *V. parahaemolyticus* in laboratory-contaminated oysters during depuration at 7, 10, 12.5 and 15 ºC. Data were reported as mean values of reductions determined from five separate oysters. The means with the same letter observed at the same depuration temperature were not significantly different ($P > 0.05$). A linear prediction for the reduction of *V. parahaemolyticus* in oysters was sketched over time.

oysters by 2-3 log CFU/g while a 72-h process was required to achieve reductions by 3-4 log CFU/g (Son and Fleet 1980).

In conclusion, reducing temperature for depuration enhanced the efficacy in reducing *V. parahaemolyticus* in oysters. However, the biological activity of oysters appeared to be minimized when oysters were exposed to temperatures below 5 ºC. Depuration of oysters at temperatures between 7 and 15 °C can be applied as a post-harvest treatment for reducing contamination of *V. parahaemolyticus*. Further studies are needed to validate the efficacy of the process in reducing *V. parahaemolyticus* accumulated naturally in oysters.

2.5 Acknowledgements

CHAPTER 3

IMPACT OF WATER SALINITY AND TYPES OF OYSTERS ON DEPURATION FOR REDUCING *Vibrio parahaemolyticus* IN PACIFIC OYSTERS (*Crassostrea gigas*)

Sureerat Phuvasate and Yi-Cheng Su

3.1 Abstract

This study investigated effects of water salinity (10, 20, 25 and 30 ppt), oyster types (diploid and triploid) and oyster sizes (extra small and medium) on depuration for reducing *Vibrio parahaemolyticus* contamination in Pacific oysters (*Crassostrea gigas*). Oysters were inoculated with a mixed culture of five clinical strains of *V. parahaemolyticus* to levels of 10^{4-6} MPN/g and depurated in UV-irradiated water in a recirculating system at 12.5 ºC for 5 days. Holding oysters (diploid, extra small) in water with a salinity of 10 ppt for 5 days resulted in about 2 $\log_{10}$ MPN/g reductions of *V. parahaemolyticus*, which were significantly smaller than those ($>3.0 \log_{10}$ MPN/g) observed in water with higher salinities (20-30 ppt). The types (diploid vs triploid) and sizes (extra small vs medium) of oysters appear to have no impact on the efficacy of depuration for reducing *V. parahaemolyticus* in oysters. However, the efficacy of depuration in reducing *V. parahaemolyticus* in oysters can be influenced by water salinity. Such a factor needs to be considered when developing a depuration process for eliminating *V. parahaemolyticus* in oysters.

Keywords: *Vibrio parahaemolyticus*, Pacific oyster, Depuration, Water salinity, Seafood safety

3.2 Introduction

Shellfish are filter-feeders and can accumulate bacteria, such as sewage-associated bacteria and human pathogens, in the growing waters, posing risks for public health when consumed raw or lightly cooked (Rippey 1994). It is known that sewage-associated bacteria can be eliminated from shellfish when they are placed in pollutant-free water in the nature (relaying) and in sterilized water under a controlled condition (depuration) (Arcisz and Kelly 1955; Buisson and others 1981; Son and Fleet 1980). Both relaying and depuration have been applied to render contaminated shellfish for safe consumption by decreasing levels of coliforms (<300 MPN/100g) or *E. coli* (<230/100g) in shellfish after the self-purification processes (European Communities 1991). However, successful removal of these bacterial indicators may not be associated with significant decrease in numbers of pathogenic bacteria such as *Vibrio* spp. (Croci and others 2002; Greenberg and others 1982).

Pathogenic *Vibrio* spp. are natural inhabitants of marine environments and have been frequently involved in many outbreaks associated with eating raw oysters (CDC 1993; CDC 1996; Daniels and others 2000a). The illnesses caused by *Vibrio parahaemolyticus* and *Vibrio vulnificus* associated with raw oyster consumption can be greatly reduced if oysters are subjected to post-harvest processes to reduce or eliminate the contamination before consumption (FAO/WHO 2005; FDA 2005). Studies have indicated potential application of depuration for reducing contamination of *Vibrio* spp. in oysters at low water temperatures, such as at 5 °C for reducing *V. parahaemolyticus* in Pacific oysters (*Crassostrea gigas*) (Su and others 2010) or at 15 °C for reducing *V. parahaemolyticus* and *V. vulnificus* in American oysters (*Crassostrea virginica*) (Chae and others 2009; Tamplin and Capers 1992). Another study reported that a flow-through depuration process with a water salinity of >30 ppt and a flow rate of 68 L/min at 25 °C was more efficient in reducing *V. vulnificus* (>4.52 $\log_{10}$ reductions) in American oysters than those subjected to the process at 25 °C with lower salinity levels and flow rates (Lewis and others 2010). On the other hand, populations of *V. vulnificus* increased in the oysters after being depurated in water with a low salinity of 9 ppt. It was believed that

exposing oysters to low water temperature or low salinity would trigger a physiological shock, resulting in less water-pumping activity and poor purification of oysters (Rowse and Fleet 1984). Our recent study observed no movement of oysters when oysters were depurated at temperatures of 3 °C or below with a small reduction of *V. parahaemolyticus* (Phuvasate and Su 2012). To date, there have been limited studies on salinities required for maintaining biological activity and removing *V. parahaemolyticus* in oysters during depuration processes. Understanding responses of oysters to water salinity will allow development of efficient depuration processes for decontaminating *V. parahaemolyticus* in oysters.

In this study, we examined movement of Pacific oysters in water of various salinities and effects of water salinity on persistence of *V. parahaemolyticus* in the oysters during depuration using UV light-disinfected (12.5 °C) seawater with salinity ranging from 10 to 30 ppt. An optimal depuration process identified from the studies was used to assess whether types and sizes of oysters would influence retention of *V. parahaemolyticus* in oysters during depuration.

3.3 Materials and methods
3.3.1 Bacterial culture preparation

Five clinical strains of *Vibrio parahaemolyticus* (10290, 10292, 10293, BE98-2029 and 027-1c1) obtained from the culture collection of the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, WA) were used in this study. Each strain was grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Spark, MD) containing 1.5% NaCl (TSB-Salt) at 37 ºC for 18-24 h. The enriched culture was streaked onto individual plates of tryptic soy agar (TSA; Difco, Becton Dickinson) supplemented with 1.5% NaCl (TSA-Salt) and incubated at 37 ºC for 18-24 h. A single colony formed on a TSA-Salt plate was transferred to TSB-Salt and incubated at 37 ºC for 4 h. Enriched cultures of *V. parahaemolyticus* were pooled into a 50-mL sterile centrifuge tube and harvested by centrifugation at 3000 x g (Sorvall RC-5B, Kendro

Laboratory Products, Newtown, CT) at 5 ºC for 15 min. Pellet cells were re-suspended in 2% salt solution to produce a culture suspension of approximately 10^{8-9} CFU/ml.

3.3.2 Oyster preparation

Raw Pacific oysters (*Crassostrea gigas*) were obtained from an oyster farm in Oregon and transported in an ice chest to the laboratory. The oysters were briefly washed with tap water and placed in a tank (45 by 30 by 30 cm; Nalgene, Rochester, NY) containing aerated artificial seawater (ASW) with a salinity of 30 ppt. The ASW was prepared by dissolving Instant Ocean Salt (Aquarium systems, Inc., Mentor, OH) in deionized water according to the manufacturer's instruction. Oysters were held in ASW at room temperature for 2-4 h to allow them to regain biological activities. Diploid extra small oysters were used for initial salinity studies. Additional studies were conducted with diploid and triploid oysters of different sizes (extra small and medium).

3.3.3 Oyster movement

Movement of oysters in ASW of various levels of salinity (5, 10, 15, 20, 25 and 30 ppt) at room temperature was determined using 6-8 oysters and monitored by a Gape Ometer (Pacific Shellfish Institute, Olympia, WA) as previously described (Phuvasate and Su 2012). Data were recorded every 5 min for up to 24 h and a change in the measurement of >0.05 cm indicated the movement of oysters.

3.3.4 Inoculation of oysters with *V. parahaemolyticus*

Oysters were subjected to a mixture of *V. parahaemolyticus* strains at a level of 10^5 CFU/ml in freshly prepared ASW (20 L). Accumulation of *V. parahaemolyticus* in oysters was conducted in aerated ASW at room temperature overnight with water being circulated (15 L/h) as previously described (Su and others 2010).

3.3.5 Depuration

Oysters inoculated with *V. parahaemolyticus* were transferred to 60 L of ASW in a laboratory-scale recirculating (1500 L/h) system equipped with a 15 W Gamma UV sterilizer (Current-USA Inc., Vista, CA) and a water chiller (Delta Star, Aqua Logic, Inc., San Diego, CA). Depuration of oysters was conducted at 12.5 ℃ with ASW of different salinities (10, 20, 25 and 30 ppt) for 5 days. All depuration experiments were conducted three times for each salinity level. Effects of types (diploid and triploid) and sizes (extra small and medium) of oysters on depuration for reducing *V. parahaemolyticus* in oysters were determined twice each with ASW of 25 ppt at 12.5 ℃.

3.3.6 Oyster size determination

The sizes of oysters were predetermined by the oyster farms. Shell length (from the longest point from anterior to posterior) and weight (meat and juice) of each oyster were measured and recorded before experiments. Results were reported as means of ten samples ± standard deviation.

3.3.7 Microbiological analysis

Densities of *V. parahaemolyticus* in oysters before and after inoculation as well as during depuration were determined using the three-tube most-probable-number (MPN) methods (BAM 2004). Five oysters were randomly picked for daily analysis. Each oyster was shucked with a sterile knife and shucked oyster meat was homogenized with an equal volume of sterile alkaline phosphate buffer saline (PBS; pH 7.4) at high speed for 1 min using a two-speed laboratory blender (Waring Laboratory, Torrington, CT). Twenty-five grams of oyster homogenate sample (1:2 dilution) was mixed with 100 ml of PBS to prepare 1:10 dilution sample suspension. Additional 10-fold dilutions of each sample suspension were prepared using PBS. All sample dilutions were individually inoculated into 3 tubes of alkaline peptone water (APW). Inoculated APW tubes were incubated at 37 ℃ for 16-18 h. A loopful (3-mm inoculating loop) of each enriched APW from positive (turbid) tubes was streaked onto thiosulfate-citrate bile salt-sucrose (TCBS;

Difco, Becton Dickinson) plates and incubated at 37 ºC for 18-24 h. Formation of colonies that were round and green or bluish on the plates were considered positive for *V. parahaemolyticus*. Levels of *V. parahaemolyticus* were determined using 3 tube MPN table by converting the number of APW tubes that were positive for *V. parahaemolyticus*. To confirm the efficacy of the UV sterilizer in inactivating *V. parahaemolyticus* cells released from oysters into recirculated water, the water was analyzed daily for *V. parahaemolyticus* by plating water samples on TCBS plates and incubated at 37 ºC for 24 h.

3.3.8 Statistical analysis

All statistical analyses were carried out by one-way Analysis of Variance (ANOVA) using SPSS 13.0 for Windows (Chicago, IL, USA) with significant differences established at a level of *P* < 0.05. Means for oyster shell lengths and oyster weights among types and sizes of oysters were compared using Tukey's test. Results from microbiological tests were transformed to log values. Tukey's test was used to compare means of the organism in oysters exposed to each salinity treatment over time. Differences in bacterial reductions among various oyster types and sizes after 5-day of treatments were also determined by Dunnett's test using extra small diploid oysters as a reference group.

3.4 Results and discussion

Movement of oysters in water with different levels of salinity is shown in Table 3.1. Frequent movement was observed in most oysters (≥75%) subjected to seawater with a salinity between 20 and 30 ppt. Large portions (50-75%) of oysters exhibited no movement at lower water salinities (15 and 10 ppt) while oysters completely stopped gapping activity when the water salinity dropped to 5 ppt. Gape distances observed for individual oysters held in water varied over time. In general, smaller gape distances (0.06 to 0.25 cm) were observed in all active oysters exposed to 10 to 15 ppt seawater than those (0.06 to 0.85 cm) for oysters in seawater with a salinity of 20 ppt or higher. It is

Table 3.1 Movement of oysters in artificial seawater of various salinities (5-30 ppt) at room temperature.

Salinity (ppt)	Total number of oysters	Number of oysters with the maximum gape (cm) range				
		No movement[a]	0.06 to 0.25	>0.25 to 0.50	>0.50 to 0.75	>0.75
5	6	6 (100%)[b]	-	-	-	-
10	8	6 (75%)	2	-	-	-
15	8	4 (50%)	4	-	-	-
20	8	2 (25%)	4	1	-	1
25	6	0 (0%)	2	2	1	1
30	8	1 (12.5%)	1	3	2	1

[a] Oyster gapping of ≤0.05 cm was considered no movement.
[b] Percentage of oysters showed no movement.

believed that oyster gapping is associated with water-pumping activity. Therefore, results of the oyster movement study suggest that a salinity of 15 ppt is the transition point for increasing biological activity of oysters and more biological activity occurs when oysters are exposed to water with a salinity of 20 ppt or higher. This corresponds to a minimum salinity (20.5 ppt) recommended in the United Kingdom for effective purification of *C. gigas* (Centre for Environment Fisheries and Aquaculture Science 2010).

Elimination of bacteria from oysters by depuration processes may be influenced by a few factors such as oyster species and environmental parameters like temperature and salinity. It has been reported that levels of *E. coli* remained constantly in American oysters when depurated in ambient seawater with a salinity of 15-ppt for 48 h while the organism was eliminated by 95, 98 and 90% at higher salinity levels of 20, 25 and 35 ppt, respectively (Blake and others 1985). Another study observed that populations of *E. coli* were slightly reduced by 1-2 log in Sydney rock oysters (*Crassostrea commercialis*) being depurated in water (18-22 °C) with lower salinities of 16-20 ppt for 48 h compared to the purification process with higher salinities (32-47 ppt) (3 log reductions) (Rowse and Fleet 1984). Long exposure (28 days) to 14-ppt seawater (15 °C) was required to completely remove 25 CFU/g of *V. vulnificus* in American oysters (Kelly and Dinuzzo 1985). This finding was supported by Kaspar and Tamplin (1993) who reported better survival (smaller reductions) of *V. vulnificus* in water samples with salinities between 5 and 15 ppt than in the water with a salinity of 25 ppt or higher incubated at 20 °C for 6 days. In addition, no *V. vulnificus* cell was detected in 38-ppt seawater after 21 days. Significant reductions (2.0-3.0 log MPN/g) of *V. vulnificus* were reported in naturally-contaminated American oysters subjected to a relaying process in waters with high levels of salinity (30-34 ppt) for 7 to 17 days (Motes and DePaola 1996). Our recent study observed greater than 3.0-log (MPN/g) reductions of *V. parahaemolyticus* in laboratory-contaminated Pacific oysters depurated in 30-ppt seawater at temperatures ranging from 7 to 15 °C for five days with no mortality (Phuvasate and Su 2012).

The effects of water salinity on efficacy of depuration for eliminating *V. parahaemolyticus* in Pacific oysters have not thoroughly investigated. In this study, we

examined depuration with water of different salinities (10, 20, 25 and 30 ppt) at 12.5 $^\circ$C for decreasing levels of *V. parahaemolyticus* in laboratory-contaminated oysters. Holding contaminated oysters in seawater with a salinity of 10 ppt resulted in small reductions of *V. parahaemolyticus* of 1.3 and 2.0 log (MPN/g) after one and five days of the process, respectively (Table 3.2). This is likely due to little or no biological activity of oysters in seawater with a low salinity of 10 ppt (Table 3.1). Greater reductions (1.7 to 2.0 log MPN/g) were observed in oysters depurated in water with higher levels of salinity (20 to 30 ppt) after one day of depuration and the reductions increased to approximately 3.0 and 3.3 log MPN/g after four and five days, respectively (Table 3.2). No mortality of oysters was observed in any of the depuration processes conducted at 12.5 $^\circ$C for 5 days.

The salinity of water in estuarine environments changes constantly and normally ranges from 20 to 30 ppt. Our studies observed no difference between *V. parahaemolyticus* reductions in oysters depurated in water with salinity between 20 and 30 ppt. Therefore, influence of types and sizes of oysters on depuration for reducing *V. parahaemolyticus* in oysters was studied with water of a salinity of 25 ppt (a middle point between 20 and 30 ppt). Information for oysters of different types and sizes are reported in Table 3.3. Regardless of ploidy, medium-sized oysters were about double in mass and had longer shell length (11.9-12.0 cm) than that (8.0-8.3 cm) of extra small-sized oysters. Both diploid and triploid oysters used in this study had similar weight and shell length for the same sizes ($P > 0.05$). The ages of oysters ranged from 12 to 18 and 24 to 30 months for the extra small- and medium-sized oysters, respectively. Triploid oysters have gained interests as an alternate type to diploid oysters due to a rapid increase in size. A 30-month study in comparison of growth between diploid and triploid Sydney rock oysters revealed that the triploid oysters reached market size at least 6 months (up to 18 months) faster than diploid oysters while maintaining better meat condition (Nell and others 1994). Harding (2007) also reported that diploid American oysters had shell height of 7.6 cm after 18 months, which took longer than triploid American oysters (14 months) and triploid Suminoe oysters (*Crassostrea ariakensis*) (13 months) to grow to the same measurement.

Table 3.2 Changes of *Vibrio parahaemolyticus* (Log_{10} MPN/g) in laboratory-contaminated oysters depurated in water with salinity ranging from 10 to 30 ppt at 12.5° C.

Day	Salinity (ppt)			
	10	20	25	30
0	5.38 ± 0.25^{a} A	4.61 ± 0.42 A	4.54 ± 0.54 A	4.61 ± 0.42 A
1	4.11 ± 0.65 B $(1.27)^{b}$	2.90 ± 0.48 B (1.71)	2.78 ± 0.50 B (1.76)	2.59 ± 0.29 B (2.02)
2	3.76 ± 0.52 BC (1.62)	2.07 ± 0.50 C (2.54)	2.34 ± 0.35 B (2.20)	2.10 ± 0.38 C (2.51)
3	3.73 ± 0.56 BC (1.65)	1.83 ± 0.57 CD (2.78)	1.80 ± 0.48 C (2.74)	1.77 ± 0.45 CD (2.84)
4	3.39 ± 0.57 C (1.99)	1.62 ± 0.58 CD (2.99)	1.57 ± 0.28 CD (2.97)	1.56 ± 0.31 DE (3.05)
5	3.31 ± 0.71 C (2.07)	1.32 ± 0.29 D (3.29)	1.28 ± 0.33 D (3.26)	1.33 ± 0.50 E (3.28)

[a] Values were reported as means ± standard deviations of fifteen oyster samples from three independent experiments for each salinity. Data with the same letter in the same column are not significantly different ($P > 0.05$).
[b] Reduction (Log_{10} MPN/g) of *Vibrio parahaemolyticus*.

Table 3.3 Weight and shell length of different types of oysters.

Type	Size [a]	Weight[b] (g)	Shell length[c] (cm)
Diploid	XS	29.7 ± 5.2[d] A	8.0 ± 0.7 A
	M	60.7 ± 13.4 B	11.9 ± 1.2 B
Triploid	XS	30.3 ± 9.2 A	8.3 ± 1.1 A
	M	63.3 ± 14.7 B	12.0 ± 1.4 B

[a] Two different sizes: XS (extra small) and M (medium).
[b] Weight of oyster meat and juice.
[c] Length (longest point from anterior to posterior).
[d] Data are means ± standard deviations of ten oyster samples. Values with the same letter in each column are not significantly different ($P > 0.05$).

Oysters of different types and sizes [4 groups: extra small (XS) diploid, XS triploid, medium (M) diploid and M triploid oysters] were compared for accumulation and depletion of *V. parahaemolyticus* by four independent depuration (12.5 °C and 25 ppt) studies (Figure 3.1). Levels of *V. parahaemolyticus* in two groups of oysters exposed to seawater containing *V. parahaemolyticus* at a level of about 10^5 CFU/ml in the same tank for 16 - 18 h were approximately 4.5 and 4.0 log for XS diploid and XS triploid oysters (Study 1), 5.5 and 5.2 log for XS diploid and M diploid oysters (Study 2), 5.0 and 4.7 log for XS triploid and M triploid oysters (Study 3) and 4.4 and 4.2 log for M diploid and M triploid oysters (Study 4). Even though initial levels of *V. parahaemolyticus* contamination in oysters were not significantly different, slightly lower levels of *V. parahaemolyticus* in medium-sized oysters (for both types) and triploid oysters (for both sizes) were observed. Power and Collins (1990) examined distribution of *E. coli* and coliphage in mussels (*Mytilus edulis*) after 48 h of exposure and reported higher densities of both contaminants in digestive tract than in the whole meat. This could possibly explain why medium-sized oysters (twice larger in weight) had less numbers of *V. parahaemolyticus* than in extra small oysters after exposure to water containing *V. parahaemolyticus*. Beatrice and others (2006) reported higher hemocyte activities (used for monitoring oyster immune capacities) in triploid oysters than in diploid oysters, which indicated that triploid oysters might be more resistant to pathogen invasion than diploid oysters.

Nevertheless, oysters, despite of types and sizes, seemed to be capable of accumulating *V. parahaemolyticus* to similar levels upon exposure to the same environment and gradually releasing *V. parahaemolyticus* during depuration over time (Figure 3.1). The highest reductions of *V. parahaemolyticus* in the contaminated oysters were observed after 5 days of depuration at 12.5 °C in water with a salinity of 25 ppt. Reductions of *V. parahaemolyticus* in M diploid, XS triploid and M triploid oysters were slightly smaller but not significantly different from those observed in XS diploid oysters (Table 3.4). La Peyre and others (2003) who investigated elimination of *V.*
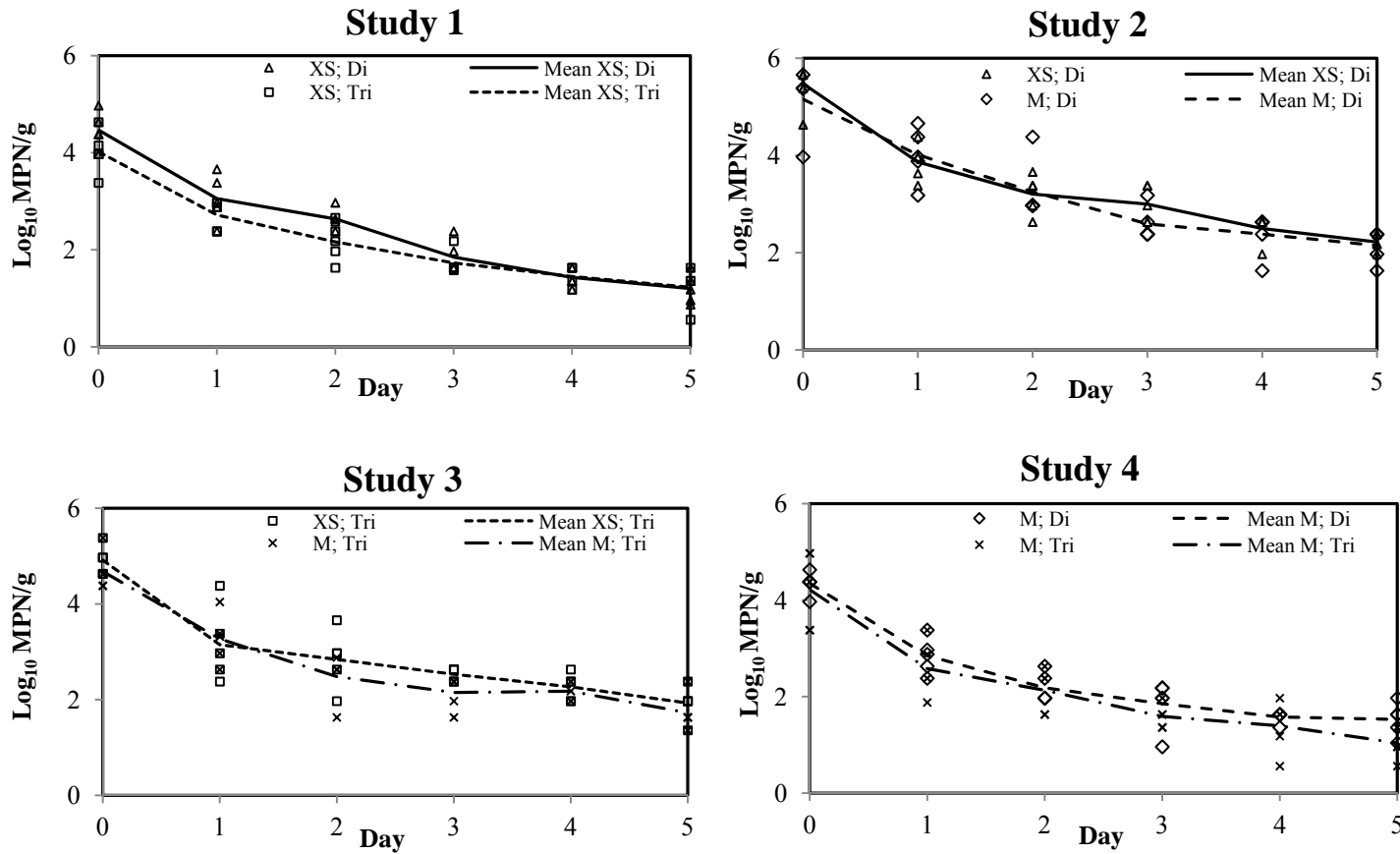
Figure 3.1 Levels of *Vibrio parahaemolyticus* in individual oysters and means of the five samples for four different type and size combinations: Δ and ⎯⎯ extra small diploid (XS; Di), ◇ and – – medium diploid (M; Di), □ and ---- extra small triploid (XS; Tri), × and – · – medium triploid (M; Tri), respectively, during 5 day of depuration at 12.5 °C with water of a salinity of 25 ppt.

Table 3.4 Reductions of *Vibrio parahaemolyticus* in oysters after 5 days of depuration (25 ppt, 12.5 °C).

Type	Size [a]	Bacterial reduction[b] (Log$_{10}$ MPN/g)
Diploid	XS	3.26 ± 0.23 A
	M	2.91 ± 0.34 A
Triploid	XS	2.90± 0.35 A
	M	3.07 ± 0.36 A

[a] XS: extra small and M: medium
[b] Means of *Vibrio parahaemolyticus* reductions were obtained from two independent trials (10 oyster samples) for each type and size combination. Values with the same letter are not significantly different ($P > 0.05$).

vulnificus density between the diploid and triploid oysters after 1-week of relaying process at 25 °C.

In summary, oyster movement studies over broad ranges of salinity (5-30 ppt) demonstrated that salinity at levels of 20 ppt or higher appear to be favorable for oysters to maintain their biological activity. Therefore, greater reductions of *V. parahaemolyticus* were observed in oysters depurated in seawater with a salinity between 20 and 30 ppt than in oysters depurated in water of a salinity of l0 ppt after 5 days at 12.5 °C. The efficacy of depuration in reducing *V. parahaemolyticus* in oysters can be influenced by water salinity but not types of oysters.

3.5 Acknowledgements

CHAPTER 4

EFFICACY OF LOW-TEMPERATURE HIGH HYDROSTATIC PRESSURE PROCESSING IN INACTIVATING *Vibrio parahaemolyticus* IN CULTURE SUSPENSION AND OYSTER HOMOGENATES

Sureerat Phuvasate and Yi-Cheng Su

4.1 Abstract

Culture suspensions of five clinical and five environmental *Vibrio parahaemolyticus* strains in 2% NaCl solution were subjected to high pressure processing (HPP) under various conditions (200 - 300 MPa for 5 and 10 min at 1.5 - 20 °C) to study differences in pressure resistance among the strains. The most pressure-resistant and pressure-sensitive strains were selected to investigate effects of low temperatures (15, 5 and 1.5 °C) on HPP (200 or 250 MPa for 5 min) to inactivate *V. parahaemolyticus* in sterile oyster homogenates. Inactivation of *V. parahaemolyticus* cells in culture suspensions and oyster homogenates was greatly enhanced by lower processing temperatures to 5 - 1.5 °C. A treatment of oyster homogenates at 250 MPa for 5 min at 5 °C decreased the populations of *V. parahaemolyticus* by 6.2 log CFU/g for strains 10290 and 100311Y11 and by >7.4 log CFU/g for strain 10292. Decreasing the processing temperature of the same treatment to 1.5 °C reduced all the *V. parahaemolyticus* strains inoculated to oyster homogenates to non-detectable (<10 CFU/g) levels. Factors including pressure level, processing temperature and time all need to be considered for developing effective HPP for eliminating pathogens from foods. A treatment of 250 MPa for 5 min at 1.5 °C can be used as a postharvest processing to eliminate *V. parahaemolyticus* contamination in oysters.

Keywords: *Vibrio parahaemolyticus,* High pressure processing, Oysters, Low temperatures, Seafood safety

4.2 Introduction

High pressure processing (HPP) technology is a non-thermal processing technology for food preservation. HPP ranging from 300 to 600 MPa and has been commercially used to inactivate spoilage and pathogenic microorganisms to extend shelf life and enhance safety of a variety of food products including jams, fruit juices, guacamole, poultry products, ready-to-eat meats and shellfish (Considine and others 2008; Gould 2000; Knorr 1999; Smelt 1998). A major advantage of applying HPP in preserving freshness and ensuring safety of food is that it does not cause detrimental effects on food qualities such as flavor, color and nutritional content (Bull and others 2004; Hayashi and others 1989; Singh and Ramaswamy 2013). Other benefits such as inactivation of enzymatic browning activity in guacamole (Palou and others 2000), meat tenderization (Sun and Holley 2010), and ease of shucking of oysters have also been reported after HPP (He and others 2002; Cruz-Romero and others 2004).

Vibrio parahaemolyticus is a Gram-negative bacterium commonly found in estuarine and marine environments and frequently isolated from seafood (Duan and Su 2005; Su and Liu 2007). This bacterium has been recognized as the leading cause of acute gastroenteritis associated with consumption of shellfish, particularly raw oysters, in the United States (FDA 2012; Daniels and others 2000a). Reducing levels of *V. parahaemolyticus* in raw oysters upon harvest and before consumption will decrease risks of illness caused by this pathogen. According to a survey in the U.S., levels of *V. parahaemolyticus* and *Vibrio vulnificus* in oysters subjected to mild heat treatment, freezing or HPP were much less (5 to 6 log) than those in unprocessed raw oysters (DePaola and others 2009). HPP (alone or combined with other treatments) has been investigated as a process for reducing *V. parahaemolyticus* and *V. vulnificus* contamination in oysters (Ye and others 2012; Ye and others 2013).

It has been reported that pathogenic *Vibrio* species are susceptible to pressure processing at 200 to 300 MPa (Berlin and others 1999). Many studies have examined effects of HPP at 200 to 350 MPa on inactivating *Vibrio* species in oysters at a temperature range of 20 - 30 °C (Calik and others 2002; Cook 2003; Styles and others

1991; Ye and others 2011). Kural and Chen (2008) studied effects of temperature (-2 to 45 °C) on pressure inactivation of *V. vulnificus* in oyster meats and reported that the bacterium was more resistant to pressure (150 - 200 MPa) at 20 - 30 °C than at <20 °C or >30 °C. They also demonstrated that a treatment at pressure levels of ≥250 MPa at -2 to 1 °C resulted in >5 log reductions of *V. vulnificus* in whole oysters within 4 min. A similar study investigating effects of temperature on pressure inactivation of *V. parahaemolyticus* reported that reductions of *V. parahaemolyticus* in oyster meats and shellstock oysters depended on the pressure applied (Kural and others 2008). A HPP treatment of oyster meats at 250 MPa for 2 min was found more effective in inactivating *V. parahaemolyticus* at 1 °C (3.1-log reduction) than at 20 °C (2.1-log reduction). On the other hand, greater reductions (>6.5 log) of this organism were observed after treatments of 300 MPa at 35 - 40 °C than at 1- 20 °C (5.3 - 5.4 log reductions). Recently, a treatment at 293 MPa for 2 min at 8 °C was validated as a post-harvest means to reduce *V. parahaemolyticus* in oysters (Ma and Su 2011). These results indicate that processing temperature needs to be considered in developing an optimal HPP for inactivating *V. parahaemolyticus* while maintaining overall quality of oysters with extended shelf life.

The objectives of this study were to determine temperature effects on the efficacy of HPP in inactivating clinical and environmental *V. parahaemolyticus* in pure culture and identify an optimal low-temperature HPP for eliminating *V. paragaemolyticus* inoculated to oyster homogenates.

4.3 Materials and methods

4.3.1 Bacterial culture preparation

Five clinical strains of *V. parahaemolyticus* obtained from the culture collection of Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, WA) and five environmental strains of *V. parahaemolyticus* isolated from oysters were used in this study (Table 4.1). Each strain was grown in trypticase soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) containing 1.5% NaCl (TSB-Salt) overnight at 37 °C. Enriched culture was streaked on thiosulfate citrate bile salt sucrose (TCBS; Becton,

Dickinson and Company, Sparks, MD) plates and incubated overnight at 37 °C. A single colony formed on the TCBS plate was picked and enriched in TSB-Salt at 37 °C for 4 h. Cells of *V. parahaemolyticus* in enriched TSB-Salt were harvested by centrifugation at 3,000 x g (Beckman J6-MI, Beckman Coulter, Inc., Brea, CA) at 5 °C for 15 min.

Table 4.1 Source of *Vibrio parahaemolyticus* strains

Strain	Virulence factor[a]	Source
Pathogenic		
10290	*tdh+, trh+*	Clinical sample, Washington
10292	*tdh+, trh+*	Clinical sample, Washington
10293	*tdh+, trh+*	Clinical sample, Washington
BE 98-2029	*tdh+, trh-*	Clinical sample, Texas
027-1c1	*tdh+, trh+*	Clinical sample, Oregon
Environmental		
100813W01	*tdh-, trh-*	Oyster, Washington
102713Y01	*tdh-, trh-*	Oyster, Oregon
090811Y02	*tdh-, trh-*	Oyster, Oregon
100311Y04	*tdh-, trh-*	Oyster, Oregon
100311Y11	*tdh-, trh-*	Oyster, Oregon

[a] Virulence factors (*tdh* and *trh*) were determined with a multiplex polymerase chain reactions (PCR) according to Bej and others (1999).

4.3.2 Preparation of *V. parahaemolyticus* in suspension and oyster homogenates

V. parahaemolyticus cells harvested from centrifugation were resuspended in sterile 2% NaCl solution or inoculated to sterilized oyster homogenates to obtain a level of approximately 10^{7-9} CFU/ml or CFU/g. To prepare oyster homogenates, oysters obtained from a local market were placed in a tank containing artificial seawater (ASW;

30 ppt) prepared from Instant Ocean Salt (Aquarium systems Inc., Mentor, OH) overnight to allow them to regain water-filtering activity. The oysters were then shucked and oyster meats and fluid were homogenized at high speed for 60 s using a two-speed laboratory blender (Waring Laboratory; Torrington, CT). The oyster homogenates were then sterilized at 121 °C for 15 min to eliminate naturally-occurring bacteria. The sterilized homogenates were blended in sterilized blender jars and then transferred to a sterile Whirl-Pak filter bag (Nasco; Modesto, CA). The pH and salinity of the sterile 2% salt solution and sterilized oyster homogenates were measured by a pH meter (SB70P SympHony, VWR International, Radnor, PA) and a conductivity meter (YSI 3100, YSI Inc., Yellow Springs, OH).

4.3.3 High hydrostatic pressure (HPP) processing of sample pouches

HPP of pure culture suspension of *V. parahaemolyticus* or oyster homogenates contaminated with *V. parahaemolyticus* was conducted using cryogenic vials (VWR; West Chester, PA). Each vial was completely filled with about 2.8 ml of sample, sealed with parafilm and then vacuum packed in a pouch (Summit Packaging, Inc; Gig Harbor, WA). Sample pouches were pressure-treated in a laboratory pressure unit (Autoclave Engineering Inc, Erie, PA) with a 2-L cylindrical vessel using 5% hydrolubric 123B water solution as a pressure-transmitting medium. Pressurization was conducted at 200, 250 and 300 MPa for 5 and 10 min at an ambient temperature (20 ± 1 °C). For HPP treatments at low temperatures, room temperature was adjusted to 15 °C and the pressure-transmitting medium was pre-chilled to 5 ± 1 or 1.5 ± 1 °C before being transferred to the pressure vessel for processing. Each experiment was performed with duplicate vials and repeated twice. Pressure come-up times were 110, 135 and 155 s at 200, 250 and 300 MPa, respectively. Release times were less than 30 s at all pressures. Processing time did not include pressure come-up and release times.

4.3.4 Microbiological analysis

After HPP treatments, vials of *V. parahaemolyticus* in 2% NaCl suspension or oyster homogenates were removed from pouches and rinsed with 70% ethanol. Viable counts of *V. parahaemolyticus* in pressure-treated and untreated samples were determined by spread plate method using trypticase soy agar (TSA; Becton, Dickinson and Company, Sparks, MD) supplemented with 1.5% NaCl. Samples were serially (10-fold) diluted with phosphate buffered saline (PBS; pH 7.4), plated (0.1 ml) on TSA-salt plates and incubated at 35-37 °C for 48 h. Colonies formed on the plates were counted and results were reported as means ± standard deviations of six samples from three independent studies (2 samples each).

4.3.5 Statistical analysis

Bacterial counts CFU/ml or CFU/g were converted to log values and statistical analyses were carried out using SPSS 13.0 for Windows (Chicago, IL) with significant differences established at $P < 0.05$. One-way Analysis of Variance (ANOVA) and Tukey's test were used to compare differences among strains and treatments. Paired-samples T-test was used to compare the difference between two treatments.

4.4 Results and discussion

4.4.1 Sensitivity of *V. parahaemolyticus* strains to pressures at ambient temperature

Inactivation of five clinical *V. parahaemolyticus* strains suspended in 2% NaCl solution (pH 6.6 - 6.7) by high pressure processes (200 - 300 MPa) at 20 °C is shown in Table 4.2. A treatment at 200 MPa for 5 or 10 min resulted in 1.1 to 4.2-log or 2.2 to 5.1-log reductions of the strains. A higher pressure treatment of 250 MPa for 5 or 10 min increased the reductions of the strains to 3.4 to 6.4-log or 5.5 to >7.1-log CFU/ml. All of the clinical strains were completely inactivated to non-detectable levels (<10 CFU/ml) by a treatment of 300 MPa for 5 min (>6.2 to >7.7-log reductions). These results indicate that the efficacy of HPP in inactivating *V. parahaemolyticus* cells varies among strains. In this study, reductions of the five clinical *V. parahaemolyticus* strains in 2% NaCl

Table 4.2 Inactivation of clinical *Vibrio parahaemolyticus* strains in 2% NaCl suspension by high pressure processing at 20 ± 1 °C

| Strain | Initial counts (Log CFU/ml) | Log_{10} reductions after pressurization | | | | | |
| | | 200 MPa | | 250 MPa | | 300 MPa | |
		5 min	10 min	5 min	10 min	5 min	10 min
10290	7.23 ± 0.10	1.13 ± 0.20[a] A	2.19 ± 0.25 A	3.35 ± 0.64 A	5.75 ± 0.39 A	>6.23	>6.23
10292	8.08 ± 0.26	4.21 ± 0.54 D	5.12 ± 0.77 D	6.36 ± 0.22 C	>7.08	>7.08	>7.08
10293	8.08 ± 0.33	2.39 ± 0.31 BC	3.05 ± 0.32 AB	4.14 ± 0.20 AB	5.51 ± 0.43 A	>7.08	>7.08
BE 98-2029	7.79 ± 0.34	1.57 ± 0.48 AB	2.55 ± 0.14 A	3.48 ± 0.65 A	5.48 ± 0.62 A	>6.79	>6.79
027-1c1	8.66 ± 0.19	3.11 ± 0.14 C	3.84 ± 0.50 C	4.86 ± 0.45B	5.82 ± 0.10 A	>7.66	>7.66

[a] Mean ± standard deviation of six samples from three studies (2 samples each). Data with the same letter in the same column are not significantly different ($P > 0.05$).

suspension after treatments of 200 - 250 MPa at 20 °C differed by up to 3.1 log CFU/ml with strains 10290 and 10292 being the most resistant and sensitive to pressure treatments, respectively.Difference in resistance to pressures has been reported among species and strains of foodborne pathogens including *V. parahemolyticus, Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* spp. (Alpas and others 1999; Berlin and others 1999; Patterson and others 1995; Styles and others 1991; Vanlint and others 2012; Wuytack and others 2002). Kural and others (2008) reported considerable differences (3 - 4 log) in pressure resistance among four *V. parahaemolyticus* strains in TSB supplemented with 2.5% NaCl after treatments of 250 - 350 MPa for 2 min at 21 °C. Berlin and others (1999) reported >6 log reductions for *V. parahaemolyticus* and *V. vulnificus* in ASW (2.3% NaCl) following treatments at 300 MPa for 5 min at 25 °C. Calik and others (2002) investigated effects of pressure against a clinical *V. parahaemolyticus* strain in alkaline peptone water (APW) supplemented with 1% NaCl and observed 6.9-log reductions after a treatment of 310 MPa for 3 min at 21 °C. A recent study of Ye and others (2013) observed 4.2-log reductions of *V. parahaemolyticus* in whole shell Eastern oysters after HPP at 250 MPa for 2 min at 22 - 24 °C. These reports clearly indicate that the efficacy of HPP in destroying microorganism can be influenced by factors, including bacterial species and strain, pressure level, suspension medium, processing time and processing temperature. Based on the results of reductions of five clinical *V. parahaemolyticus* strains by pressure treatments (200, 250, and 300 MPa) for 5 and 10 min at 20 °C, HPP at 200 and 250 MPa for 5 min were selected for studying effects of low-temperature (15, 5 and 1.5 °C) HPP on the processes for inactivating *V. parahaemolyticus* in pure culture suspension and oyster homogenates.

4.4.2 Effect of low-temperature on high pressure processing for inactivation of *V. parahaemolyticus* in 2% NaCl suspension

Inactivation of five clinical and five environmental *V. parahaemolyticus* strains by pressure treatments of 200 and 250 MPa for 5 min at three temperatures (15, 5 and 1.5 °C) are shown in Table 4.3. HPP of 200 MPa for 5 min at 15 °C reduced populations of

the five clinical strains by 1.3 to 4.1 log CFU/ml, which were similar to the reductions observed at 20 °C (Table 4.2). Compared with the clinical strains, slightly greater reductions in populations (2.3 to 3.5 log CFU/ml) were observed for five environmental strains after the treatment of 200 MPa for 5 min at 15 °C. Reducing the processing temperature of HPP (200 MPa for 5 min) from 15 to 5 °C significantly increased reductions of the clinical strains, except 10290, to 1.4 - 4.8 log CFU/ml. However, no significant increase in reductions was observed for all environmental strains after the same treatment (200 MPa for 5 min at 5 °C). Similar patterns of pressure resistance were observed for all five clinical strains treated with 250 MPa for 5 min at 15 and 20 °C (Table 4.2). Significant increases in reductions were observed for all clinical and environmental strains when the temperature of treatments (250 MPa) decreased from 15 to 5 °C. The increased reductions of clinical strains ranged from 6.1 to >7.45 log CFU/ml with a complete inactivation being observed for strain 10292. Similarly, reductions of all environmental strains increased to 6.2 - 7.1 log CFU/ml after 250 MPa treatment at 5 °C.

Further decrease of the processing temperature to 1.5 °C significantly increased reductions of all clinical and environmental strains when compared with reductions observed at 5 °C. The reductions in bacterial populations after a treatment of 200 MPa at 1.5 °C for 5 min ranged from 2.6 to 6.8 log CFU/ml for the clinical strains and from 3.6 to 4.3 log CFU/ml for the environmental strains. A treatment of 250 MPa at 1.5 °C for 5 min resulted in >6.5-log reduction for strain 10290 and >7.0-log reductions for others. These data demonstrate that the efficacy of HPP in inactivating *V. parahaemolyticus* was significantly enhanced at cold temperatures (1.5 - 5 °C). Cook (2003) reported that *V. parahaemolyticus* O3:K6 strains were more pressure-resistant than non O3:K6 strains in PBS when treated at 250 MPa and 10 °C. In this study, strain BE98-2029 (O3:K6) became more sensitive than a few non O3:K6 strains (10290 and 10293) to pressure treatments (200 and 250 MPa) at 5 °C. However, differences in pressure resistance among clinical and environmental strains of *V. parahaemolyticus* were also observed even after a treatment at 1.5 °C. Similar to the observation obtained at 20 °C (Table 4.2), strains 10290 and 10292 remained as the most pressure-resistant and the most pressure-

Table 4.3 Inactivation of clinical and environmental *Vibrio parahaemolyticus* in 2% NaCl suspensions by high pressure treatments of 200 and 250 MPa for 5 min at 15, 5 and 1.5 °C

Strain	Initial counts (Log$_{10}$ CFU/ml)	Log$_{10}$ CFU/ml reductions after pressurization					
		200 MPa			250 MPa		
		15 °C	5 °C	1.5 °C	15 °C	5 °C	1.5 °C
10290	7.48 ± 0.16	1.25 ± 0.10 [a] Aa	1.40 ± 0.14 Aa	2.60 ± 0.28 Ab	3.93 ± 0.30 Aa	6.10 ± 0.18 Ab	>6.48
10292	8.45 ± 0.07	4.07 ± 0.22 Fa	4.84 ± 0.25 Gb	6.80 ± 0.19 Dc	6.22 ± 0.31 Fa	>7.45 Eb	>7.45
10293	8.20 ± 0.13	2.11 ± 0.27 Ba	2.77 ± 0.19 CDb	3.98 ± 0.30 BCc	4.15 ± 0.10 ABCa	6.63 ±0.23 BCDb	>7.20
BE 98-2029	8.13 ± 0.04	1.99 ± 0.23 Ba	3.50 ± 0.20 EFb	4.18 ± 0.29 BCc	4.04 ± 0.13 ABa	6.40 ± 0.32 ABCb	>7.13
027-1c1	8.62 ± 0.03	3.12 ± 0.18 DEa	3.71 ± 0.05 Fb	4.22 ± 0.23 Cc	4.84 ± 0.15 DEa	6.17 ± 0.08 ABb	>7.62
100813W01	8.33 ± 0.19	2.97 ± 0.29 DEa	2.94 ± 0.29 CDEa	4.02 ± 0.26 BCb	4.59 ± 0.29BCDa	7.07 ± 0.11 DEb	>7.30[b]
102713Y01	8.45 ± 0.22	2.64 ± 0.20 CDa	2.68 ± 0.41 BCa	4.26 ± 0.23 Cb	4.78 ± 0.21 CDEa	7.10 ± 0.17 DEb	>7.29[b]
090811Y02	8.33 ± 0.06	3.48 ± 0.15 Ea	3.33 ± 0.43 DEFa	4.04 ± 0.19 BCb	5.33 ± 0.37 Ea	7.10 ± 0.31 DEb	>7.21[b]
100311Y04	8.10 ± 0.09	3.27 ± 0.07 DEa	3.36 ± 0.12 DEFa	4.33 ± 0.34 Cb	5.27 ± 0.15 Ea	6.81 ± 0.19 CDb	>6.95[b]
100311Y11	8.27 ± 0.20	2.28 ±0.34 BCa	2.14 ± 0.14 Ba	3.57 ± 0.29 Bb	4.23±0.25ABCDa	6.24 ± 0.28 ABb	>7.22[b]

[a] Mean ± standard deviation of six samples from three studies. Data in the same column having the same upper case and data in the same row within the same pressure level having the same lower case letter are not significantly different (*P* > 0.05).
[b] Counts of *V. parahaemolyticus* in at least one of the replicates were >10 CFU/ml.

sensitive ones among the clinical strains while strain 100311Y11 was the most pressure-resistant one among the environmental strains. These three strains were selected to study effects of low-temperatures on HPP in inactivating *V. parahaemolyticus* in oyster homogenates.

4.4.3 Effect of low-temperature on high pressure processing for inactivating *V. parahaemolyticus* in oyster homogenates

Reductions of *V. parahaemolyticus* strains 10290, 10292 and 100311Y11 inoculated to sterilized oyster homogenates (pH of 6.3 - 6.4) with a salinity of 15 ± 1 ppt by pressure treatments of 200 MPa for 5 min at 15, 5 and 1.5 °C are shown in Table 4.4. A treatment of 200 MPa for 5 min at 15 °C slightly reduced populations of strains 10290 (0.8 log CFU/g) and 100311Y11 (1.1 log CFU/g) in oyster homogenates while a much greater reduction of 3.4 log CFU/g was observed for strain 10292. These reductions in oyster homogenates were smaller than those (1.3 log CFU/g for 10290, 2.3 log CFU/g for 100311Y11 and 4.1 log CFU/g for 10292) observed in 2% NaCl solution from the same treatment (Table 4.3). The smaller reductions of *V. parahaemolyticus* populations observed in oyster homogenates than in culture suspension after the treatment of 200 MPa for 5 min at 15 °C might be due to a protective effect from oyster components (Calik and others 2002). A study of pressure treatments of 276 and 310 MPa at 21 °C for inactivating a *V. parahaemolyticus* O3:K6 strain in whole oysters and PBS also reported that the processing times required to reduce 1.0 log unit of *V. parahaemolyticus* were three times greater in whole oysters than in PBS by the treatments (Koo and others 2006).

Similar to the pressure treatments of *V. parahaemolyticus* cells in culture suspension, a decrease of processing temperature of HPP (200 MPa for 5 min) from 15 to 5 °C significantly increased reductions of strains 10290, 100311Y11 and 10292 to 1.6, 2.4 and 4.6 log CFU/g, respectively. Further decrease of the processing temperature from 5 to 1.5 °C for the same pressure treatment increased reductions of strains 10290, 100311Y11 and 10292 to 2.3, 3.5 and 6.5 log CFU/g, respectively. The reductions of the three *V. parahaemolyticus* strains in oyster homogenates (Table 4.4) were similar to those observed in culture suspension (2.6 log CFU/ml for 10290, 3.6 log CFU/ml for

Table 4.4 Inactivation of *Vibrio parahaemolyticus* in oyster homogenates by high pressure treatments of 200 and 250 MPa for 5 min at 15, 5 and 1.5 °C

| Strain | Initial counts (Log$_{10}$ CFU/ml) | Log$_{10}$ CFU/ml reductions after pressurization | | | | | |
| | | 200 MPa | | | 250 MPa | | |
		15 °C	5 °C	1.5 °C	15 °C	5 °C	1.5 °C
10290	7.43 ± 0.14	0.77 ± 0.12[a] a	1.55 ± 0.23 b	2.25 ± 0.30 c	4.21 ± 0.16 a	6.18 ± 0.25 b	>6.43
10292	8.36 ± 0.11	3.37 ± 0.11 a	4.56 ± 0.34 b	6.46 ± 0.22 c	6.13 ± 0.31a	>7.36 b	>7.36
100311Y11	8.22 ± 0.16	1.05 ± 0.14 a	2.38 ± 0.14 b	3.45 ± 0.26 c	4.30 ± 0.17 a	6.15 ± 0.20 b	>7.22

[a] Values are mean ± standard deviation of six samples from three independent studies. Data with the same letter in the same row with the same pressure level are not significantly different ($P > 0.05$).

100311Y11 and 6.8 log CFU/ml for 10292) (Table 4.3). These results demonstrate again that the efficacy of HPP in inactivating *V. parahaemolyticus* in oysters can be enhanced by decreasing processing temperatures to 1.5 °C.

Increasing the pressure of treatments at 15°C from 200 to 250 MPa resulted in significant increases in reductions of strains 10290 (4.2 log CFU/g), 100311Y11 (4.3 log CFU/g) and 10292 (6.1 log CFU/g) from 0.8, 1.1 and 3.4 log CFU/g, respectively, observed from the treatments of 200 MPa (Table 4). Decreasing the processing temperature of 250 MPa from 15 to 5 °C further increased the reductions to 6.2 log CFU/g for strains10290 and 100311Y11 with a complete inactivation (>7.3 log CFU/g) for strain10292 (Table 4.4). A treatment of 250 MPa for 5 min at 1.5 °C reduced the populations of all three *V. parahaemolyticus* strains in oyster homogenates to non-detectable levels (<10 CFU/g) with >6.4, >7.2 and >7.4 log reductions being observed for strains 10290, 100311Y11 and 10292, respectively. These results further demonstrate that greater reductions of *V. parahaemolyticus* cells in oysters by HPP can be achieved by conducting the processes at low temperatures (1.5 - 5 °C). Greater reductions of *V. parahaemolyticus* in oyster meats were also reported after a treatment of 250 MPa for 5 min at -2 °C (5.9 log) than at 20 °C (4.4 log) (Kural and others 2008). Another study observed reductions of *V. vulnificus* in oyster meats increased from 0.5 to 4.7 log after treatments at 150 MPa for 4 min by lowering the processing temperature from 20 to -2 °C (Kural and Chen 2008). While increased reductions of *V. parahaemolyticus* in oyster homogenates were reported after pressure treatments at 1.5 °C, difference in pressure resistance was still observed among the *V. parahaemolyticus* strains subjected to the treatments. Strains 10290 and 100311Y11 were more pressure-resistant than strain 10292 when oyster homogenates were subjected to treatments of 250 MPa at 15 and 5 °C (Table 4.4). Nevertheless, HPP of 250 MPa for 5 min at 1.5 °C completely eliminated all three strains of *V. parahaemolyticus* inoculated to oyster homogenates.

In conclusion, this study demonstrated variations in pressure resistance among clinical and environmental strains of *V. parahaemolyticus*. The efficacy of HPP in inactivating *V. parahaemolyticus* in oysters can be enhanced by lowering the operation

temperature. Pressure level, processing temperature and time are parameters to be considered in developing the optimal HPP for inactivating pathogens in foods. A treatment of 250 MPa for 5 min and 1.5 °C was sufficient to inactivate >6.5 and >6.4 log of *V. parahaemolyticus* in suspension and oyster homogenates, respectively. A 5-min treatment of 250 MPa at 1.5 °C can be applied as a post-harvest treatment for producing oysters free of *V. parahaemolyticus* for raw consumption.

4.5 Acknowledgements

CHAPTER 5

ALTERATIONS OF CELL STRUCTURE AND CELLULAR PROTEINS OF
Vibrio parahaemolyticus BY HIGH PRESSURE PROCESSING

Sureerat Phuvasate and Yi-Cheng Su

5.1 Abstract

Effects of high pressure processing (HPP) on changes of cell morphology and cellular proteins were investigated using two *Vibrio parahaemolyticus* strains 10290 and 10292 (more pressure sensitive than strain 10290) subjected to pressure treatments (200 or 300 MPa for 5 min) at 20 °C. Cells with or without pressure treatments were studied for structural damage by leakage of internal cellular substances and scanning electron microscopy (SEM) and for cellular proteins profiles by total sulfhydryl contents and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Increase in leakage of cellular substances was observed as the pressure of a treatment increased. Scanning electron microscopy images revealed cell damage of both *V. parahaemolyticus* strains after the pressure treatments, particularly at 300 MPa for 5 min. Total protein and sulfhydryl contents in cellular protein extracts of both strains decreased as the pressure of a treatment increased. SDS-PAGE analysis of cellular proteins extracted from *V. parahaemolyticus* cells after the pressure treatments revealed changes of cellular protein profiles with a few protein fragments of 150 - 250 kDa being affected most. These data suggest that inactivation of *V. parahaemolyticus* cells by high pressure processing is associated with cell membrane damage and changes of cellular protein profiles, which appear to be dependent on the strain and pressure applied in a treatment.

Key words: *Vibrio parahaemolyticus*, High hydrostatic pressure, Cell damage, Protein profiles

5.2 Introduction

High hydrostatic pressure processing (HPP) is a non-thermal process which can be utilized for food preservation. The process inactivates microorganisms and destroys enzymatic activity in food at ambient and low temperatures without breaking covalent bonds. Therefore, food products processed with high pressures generally retain fresh-like attributes as well as nutritional and bioactive components (Hayashi and others 1989; Styles and others 1991). The efficacy of HPP in inactivating microorganisms varies depending mainly on the conditions of pressure treatments (pressure, time and temperature) and can be influenced by other factors such as microbial types and cell growth phase at the time of pressurization (Pagán and Mackey 2000; Alpas and others 1999; Berlin and others 1999; Ye and others 2011). In general, yeasts, molds and bacterial vegetative cells are sensitive to pressure below 700 MPa while bacterial spores are more pressure-resistant and may survive after pressurization above 1000 MPa (Arroyo and others 1997; Hoover and others 1989; Sale and others 1970).

Under a pressure condition, a number of changes may occur in microbial cells including alterations in cell membrane, morphology and biochemical reactions (Hoover and others 1989; Patterson 2005). Alteration of cell membrane is a common phenomenon observed in bacterial cells after high-pressure treatments. Many studies have reported relationship between loss of bacterial cell viability and cell membrane damage which can be determined by a decrease in intracellular ATP (Wouters and others 1998), leakage of UV-absorbing substances (Shimada and others 1993), increased uptake of florescent dyes (Ritz and others 2002; Pagán and Mackey 2000), or detection of intracellular proteins (Malone and others 2002) from pressure-treated cells. Tholozan and others (2000) reported a progressive decrease of intracellular potassium and ATP in *Listeria monocytogenes* and *Salmonella* Typhimurium with the increase of pressure.

Numerous studies on morphological changes of microorganisms induced by high pressures have been investigated by electron microscopy (Durães-Carvalho and others 2012; Kaletunç and others 2004; Ritz and others 2002; Sato and others 1995; Shimada and others 1993). Tholozan and others (2000) observed an increase in cell invagination

for *S.* Typhimurium after a treatment at 325 MPa for 10 min. A later study also found an increase in number of pimples and swellings for *L. monocytogenes* cells after a pressure treatment of 400 MPa for 10 min. Ritz and others (2001) reported bud scars on cell surface of *L. monocytogenes* after the same pressure treatment (400 MPa for 10 min). Kaletunç, and others (2004) studied effects of pressure treatments (250 and 500 MPa for 10 min at 35 °C) on structural changes of *Leuconostoc mesenteroides* and observed more blister-like formation on the surface of *L. mesenteroides* cells exposed to 500 MPa than to 250 MPa. .However, a similar study of Kalchayanand and others (2002) observed no difference in size, shape and surface structure of *L. mesenteroides* cells after a treatment at 345 MPa for 5 min. This suggests that effects of pressures on cell structure vary depending on the pressure-time combination.

It has been reported that HPP can destroy hydrophobic and electrostatic interactions while hydrogen bonding is not affected by pressures (Rivalain and others 2010). Change in membrane-bound enzymes and membrane composition has been reported due to protein denaturation (Tholozan and others 2000; Wouters and others 1998). Ritz and others (2000) observed changes in cytoplasmic and outer membrane proteins of *S.* Typhimurium cells subjected to pressure treatments (350, 400 and 600 MPa). Recently, a study reported changes in protein profiles of *V. parahaemolyticus* cells after pressure treatments of 200 - 300 MPa at room temperature (25 °C) for 10 min (Wang and others 2013). However, the mechanism of HPP in inactivating *V. parahaemolyticus* cells has not been fully elucidated.

The objective of this study was to investigate alterations of cell morphology and changes in cellular proteins of two clinical *V. parahaemolyticus* strains 10290 and 10292 (less pressure-resistant than strain 10290) subjected to pressure treatment at 200, 250 and 300 MPa for 5 and 10 min at 20 ± 1 °C. Leakage of intracellular materials from *V. parahaemolyticus* cells into culture suspension after treatments was used to assess membrane integrity. Alteration of cell morphology was examined with scanning electron microscopy (SEM) imaging. Protein and sulfhydryl (SH) contents, protein profiles and protease activity of cellular proteins obtained from untreated and pressure-treated cells

were analyzed to comprehend the mechanism of HPP on inactivating *V. parahaemolyticus* cells.

5.3 Materials and methods

5.3.1 *V. parahaemolyticus* culture preparation

Two clinical *V. parahaemolyticus* strains 10290 and 10292 obtained from the culture collection of Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, WA) were used in this study. Strain 10290 was found more pressure-resistant than strain 10292 under treatments of 200 and 250 MPa for 5 and 10 min at 20 °C (unpublished data). Each strain was grown in trypticase soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) containing 1.5% NaCl (TSB-Salt) and incubated overnight at 37 °C. The enriched culture was streaked onto thiosulfate citrate bile salt sucrose (TCBS; Becton, Dickinson and Company, Sparks, MD) plates and incubated overnight at 37 °C. A single colony on a TCBS plate was picked and enriched in TSB-Salt at 37 °C for 4 h to allow growth to reach an early stationary phase. Cells of *V. parahaemolyticus* were harvested by centrifugation at 3000 x g (Beckman J6-MI, Beckman Coulter, Inc., Brea, CA) for 15 min at 5 °C. Cell pellet was re-suspended in 2% NaCl solution to prepare a culture suspension of approximately 10^8 CFU/ml.

5.3.2 High-pressure treatments

V. parahaemolyticus cells in 2% NaCl suspension were transferred to a sterile cryogenic vial (VWR; West Chester, PA). Each vial (2.8 ml) was filled with the cell suspension, sealed with parafilm and snap-on cap before being vacuum-packed in a pouch (Summit Packaging, Inc; Gig Harbor, WA). Sample pouches were pressure-treated with a laboratory pressure unit (Autoclave Engineering Inc, Erie, PA) equipped with a 2-L cylindrical vessel using 5% hydrolubric 123B water solution as a pressure-transmitting medium. Pressurization was conducted at 200, 250 and 300 MPa for 5 and 10 min at an ambient temperature of 20 ± 1 °C. All treatments were repeated twice.

5.3.3 Analysis of nucleic acid and protein contents in cell suspension

Cell suspension (1.5 ml) of *V. parahaemolyticus* with and without pressure treatments were transferred to a sterile microcentrifuge (1.8 ml) tube (VWR; Radnor, PA) and centrifuged at 10,000 x g (Biofuge Fresco, Kendro Laboratory Products, Germany) for 15 min at 5 °C. The supernatant was collected and examined for nucleic acid and protein contents by measuring the absorbance at 260 and 280 nm, respectively, using Biophotometer plus (Eppendorf AG, Hamburg, Germany). Protein concentration in the supernatant was also determined by the method of Bradford (1976) using Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA) at 595 nm with bovine serum albumin as a reference.

5.3.4 Scanning electron microscopy (SEM) analysis of *V. parahemolyticus* cells with and without pressure treatments

Cells of *V. parahaemolyticus* in 2% NaCl suspension without and with pressure treatments (200 or 300 MPa for 5 min) were harvested by centrifuged at 10,000 x g for 15 min at 5 °C. Cell pellet was preserved in a fixative agent (2.5% glutaraldehyde and 1% paraformaldehyde in a 0.1 M cacodylate buffer) and stored at 4 °C prior to SEM analysis. The fixed cells were rinsed with 0.1 M cacodylate buffer, dehydrated through a series of acetone (10, 30, 50, 70, 90 and twice in 100%) and critical point dried in CO_2 medium. The cells were then coated with gold and palladium (60:40 ratio) for imaging at high vacuum and examined in a Quanta 600 FEG field emission scanning electron microscope (FEI Inc., Hillsboro, OR).

5.3.5 Extraction of proteins from cell pellet

Cell pellets of pressure-treated and untreated samples after centrifugation were mixed with B-PER bacterial protein extraction reagent (Thermo Fisher Scientific Inc., Rockford, IL) containing lysozyme (50 mg/ml) and DNase I (2500 U/ml), according to manufacture's instructions. The cell mixture was centrifuged at 15,000 g for 5 min at 5 °C. The supernatant was collected for analysis of soluble proteins. Proteins, which could

not be extracted by the reagent and remained in the precipitate after centrifugation, were further extracted with 4 M urea (Amresco, Solon, OH). Concentrations of soluble proteins and urea-extracted proteins were determined with the Bradford Protein Assay as previously described.

5.3.6 Analysis of cellular protein profiles by SDS-PAGE

Cellular proteins (5-10 µg) extracted from cell pellets with B-PER bacterial protein extraction reagent or 4 M urea were analyzed by SDS-PAGE (Laemmli 1970) with 12% acrylamide in separating gel and 4% acrylamide in stacking gel under reducing (presence of beta-mercaptoethanol; BME) and non-reducing (presence of N-ethylmaleimide; NEM, Sigma-Aldrich, St. Louis, MO) conditions. Briefly, protein extracts were mixed 1:1 with loading buffer (containing either BME to break disulfide bonds or NEM to protect disulfide bonds). Protein samples, except urea-extracted samples, were heated at 95 °C for 4 min and then loaded onto wells of the stacking gel. Electrophoresis was performed in a mini-Protean III cell (Bio-Rad Laboratories, Hercules, CA) at 60 mA and 150 V for 50 min. Gels were stained using Commassie Brilliant Blue R-250 and intensity of protein bands were analyzed by Quantity One 1-D software for Gel Doc XR (Bio-Rad Laboratories, Milan, Italy). All chemicals and reagents used for SDS-PAGE were from Bio-Rad Laboratories, Inc., Hercules, CA) unless specified.

5.3.7 Determination of total sulfhydryl (SH) contents in protein extracts

Total SH contents in cellular protein extracts were determined by the method of Hamada and others (1994) using Ellman's reagent [5,5'dithiobis (2-nitrobenzoic acid); DTNB; Sigma-Aldrich, St. Louis, MO]. This method allows reactions between protein SH groups and DTNB at neutral and alkali pH, resulting in formation of the thionitrophenylated protein and a yellow thionitrophenylate anion (Kalab 1970). Briefly, each cellular protein extract (100 µl) was mixed with 8M urea in 0.2 M Tris buffer (1.5 ml, pH 7.0) and 10 mM DNTB (37.5 µl). The mixture was incubated in a water bath (40

°C) for 15 min to allow proteins to unfold and all SH groups to be accessible to DTNB. Absorbance of the mixture was measured at 412 nm using a UV-Vis spectrophotometer (UV 2401 PC, Shimadzu Corp., Kyoto, Japan). A molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ for the thionitrophenylate anion at 412 nm was used to calculate total SH groups.

5.3.8 Analysis of protease activity

Cellular protein extracts obtained from *V. parahaemolyticus* cells after pressure treatments of 200, 250 and 300 MPa for 10 min were tested for protease activity with the QuantiClave Protease Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL) using trypsin as a standard according to the manufacture's protocols. This assay detects protease activity based on the reaction of primary amines formed through proteolysis of succinylated casein (substrate) and trinitrobenzenesulfonic acid (dye), resulting the formation of orange-yellow products. A blank for each sample was made to correct the effect of amine (from general proteins). The color intensity was measured at 450 nm. Protease activity in 2% NaCl solution and in B-PER extraction reagent was also determined.

5.3.9 Statistical analysis

All experiments were repeated twice. Resulted were reported as mean values ± standard deviations calculated from data of three independent experiments. One-way Analysis of Variance (ANOVA) and Tukey's test were used to compare differences among means using SPSS 13.0 for Windows (Chicago, IL). Significant differences were established at $P < 0.05$.

5.4 Results and discussion

5.4.1 Analysis of nucleic acid and protein contents in cell suspension after pressure treatments

Nucleic acid and protein contents in the culture supernatant of *V. parahaemolyticus* 10290 and 10292 cells in 2% NaCl solution after various pressure

treatments at 20 °C are reported in Fig. 5.1. Supernatant of *V. parahaemolyticus* 10290 and 10292 culture suspension without any pressure treatments had a similar absorbance at 260 and 280 nm with no protein being detected by the Bradford Protein Assay. A treatment of strain 10290 cells at 200 MPa for 5 min resulted in a slight increase in absorbance at 260 and 280 nm with a very small amount of protein (0.01 mg/ml) being detected in the culture supernatant, which were not different from those observed from the controls (Fig. 5.1A). The absorbance values at both 260 and 280 nm as well as protein contents of the culture supernatant of *V. parahaemolyticus* 10290 cells increased as the pressures of treatments increased. The highest values of absorbance at 260 nm (1.8), 280 nm (1.1) and protein content (0.10 mg/ml) were recorded after a treatment of 300 MPa for 10 min. A similar trend in increased absorbance values and protein contents was observed for strain 10292 as the pressures of treatments increased (Fig 5.1B). However the increases in the absorbance values and protein contents of culture supernatant of strain 10292 were much greater than those observed for strain 10290 after each pressure treatment. The values of absorbance at 260 nm (3.6), 280 nm (1.9) and protein content (0.17 mg/ml) obtained from the culture supernatant of *V. parahaemolyticus* 10292 after a treatment of 300 MPa for 10 min were about twice the values of those observed for strain 10290. Shimada and others (1993) reported that absorbance values at 260 nm increased to 2.0 and 3.0 after the cells of yeast *Saccharomyces cerevisiae* were treated with 250 and 300 MPa for 10 min at 25 °C, respectively.

Studies of effects of high pressure processing on microbial cells generally demonstrate greater cell membrane damage and higher lethality with increases in pressures (Tholozan and others 2000; Durães-Carvalho and others 2012; Pagán and Mackey 2000). Increased contents of nucleic acids and proteins in the culture suspension of both *V. parahaemolyticus* strains 10290 and 10292 after pressure treatments indicated cell disruption under pressurized conditions (Fig. 5.1). In addition to cell disruption, variation in pressure resistance among *V. parahaemolyticus* strains was noted with significantly higher contents of both nucleic acids and proteins being detected in the culture supernatant of strain 10292 than that of strain 10290 after the same pressure
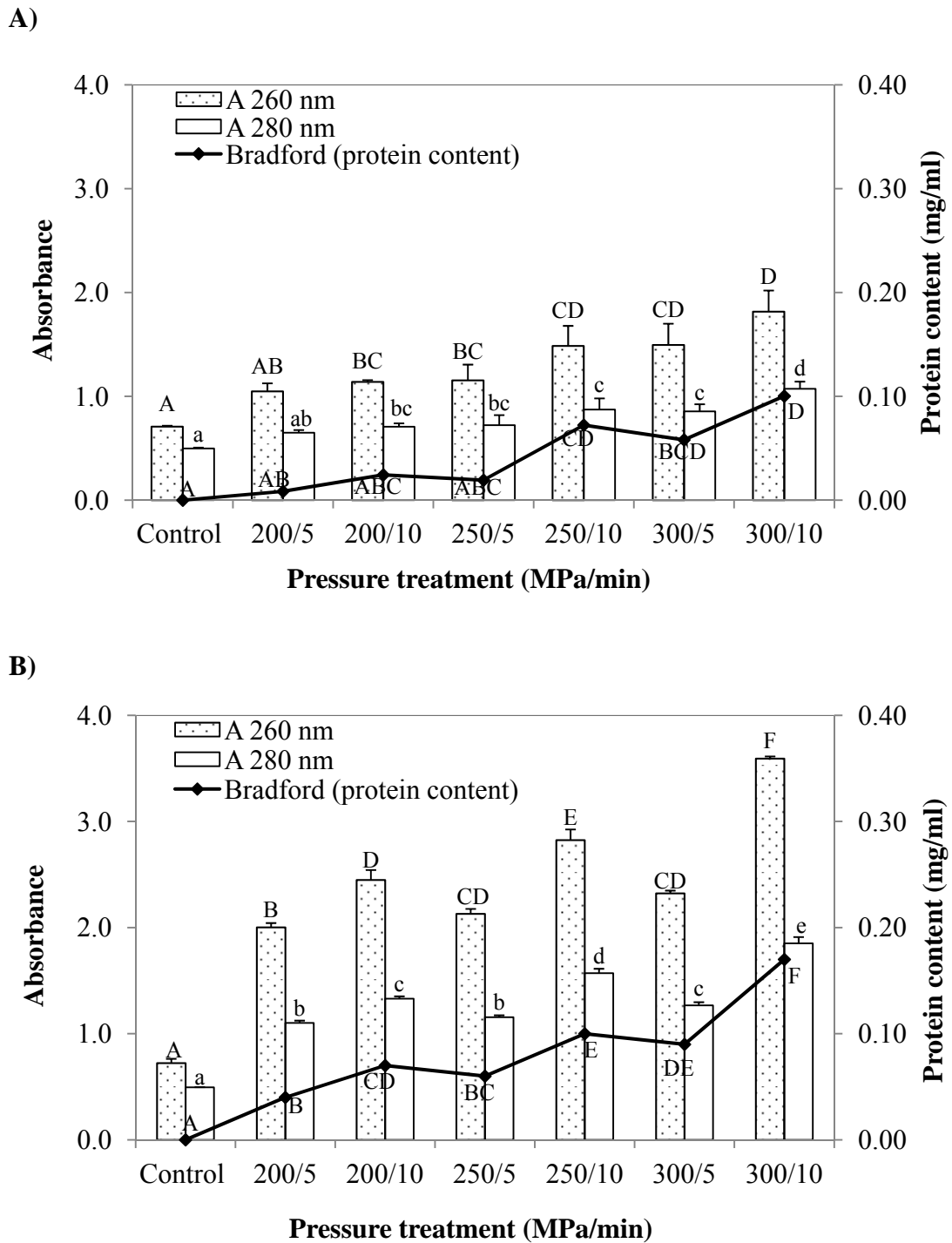
A)

B)

Fig 5.1 Nucleic acid (A 260 nm) and protein (A 280 nm and Bradford Assay) contents in culture supernatant of pressure-treated *Vibrio parahaemolyticus* strain 10290 (A) and strain 10292 (B). Values are means of three independent experiments. Data with the same letter in the same measurement are not significantly different ($P > 0.05$).

treatment. These results correspond to our previous observation of greater reductions of strain 10292 than strain 10290 after treatments of 200 and 250 MPa for 5 and 10 min at 20 °C (unpublished data) and confirm that strain 10290 was more resistant to pressure treatments than strain 10292. The difference in pressure resistance among bacterial strains may be due to different protein components in the membrane (Benito and others 1999).

5.4.2 SEM of *V. parahaemolyticus* cells

V. parahaemolyticus is a curved, rod-shaped Gram-negative bacterium with average dimensions of 0.6 to 0.9 by 1.6 to 3.1 μm (Jiang and Chai 1996). Electron micrographs of *V. parahaemolyticus* cells before pressure treatments (200 MPa for 5 min and 300 MPa for 5 min) showed cells of *V. parahaemolyticus* 10290 and 10292 were rod-shaped with a relatively smooth surface (Fig. 5.2A and 5.2D). Both strains retained their morphological characteristics (Fig. 5.2B and 5.2E) with limited cell disruption being observed for strain 10292 (Fig. 5.2E) after a treatment of 200 MPa for 5 min. However, significant structural changes, including irregular shape, rapture of cells and presence of blebs, of cells of both *V. parahaemolyticus* strains were observed after a treatment of 300 MPa for 5 min (Fig. 5.2C and 5.2F). These observations indicate that cells of *V. parahaemolyticus* can be significantly disrupted and destroyed under a pressure treatment of 300 MPa for 5 min. This corresponds to a previous study that a pressure treatment of 293 MPa for 2 min at 8 °C was capable of inactivating *V. parahaemolyticus* in oysters by >3.52-log MPN/g (Ma and Su 2011).

5.4.3 Protein contents and SH groups in cellular extracts after pressure treatments

Cellular proteins extracted from cell pellet of two *V. parahaemolyticus* strains without a pressure treatment had similar protein (1.86 mg/ml for strain 10290 and 1.96 mg/ml for strain 10292) and SH contents (124 mM/ml for strain 10290 and 120 mM/ml for strain 10292) (Fig. 5.3). The protein and SH contents in the extracts of both strains decreased as the pressure of a treatment increased. Protein contents of *V. parahaemolyticus* 10290 decreased from 1.86 to 1.75 mg/ml (decreased by 6%) and 1.38
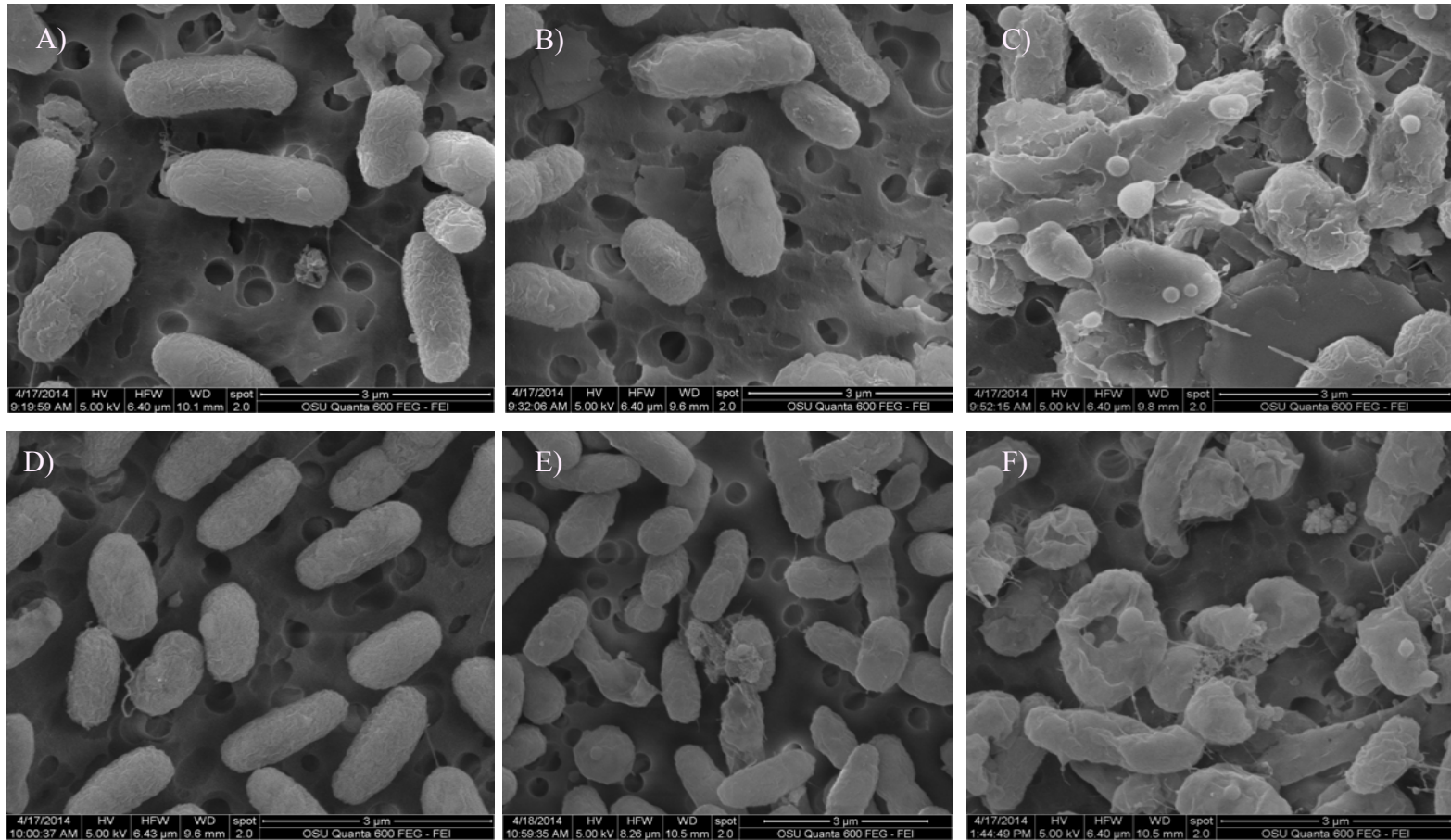
Fig 5.2 SEM micrographs of *Vibrio parahaemolyticus* cells before and after pressure treatments at 20 °C. *V. parahaemolyticus* 10290 untreated cells (A), cells treated with 200 MPa for 5 min (B) and cells treated with 300 MPa for 5 min (C). *V. parahaemolyticus* 10292 untreated cells (D), cells treated with 200 MPa for 5 min (E) and 300 MPa for 5 min (F).

A)

B)

Fig 5.3 Protein (bars) and sulfhydryl contents (◆) in cell pellet extracted with B-PER protein extraction reagent after pressure treatments. *Vibrio parahaemolyticus* strain 10290 (A) and strain 10292 (B). Values are means of three independent experiments. Data with the same letter in the same measurement are not significantly different (*P* > 0.05).

mg/ml (decreased by 26%) after a treatment of 200 and 250 MPa for 5 min, respectively (Fig. 5.3A). On the other hand, a rapid decrease in protein contents from 1.96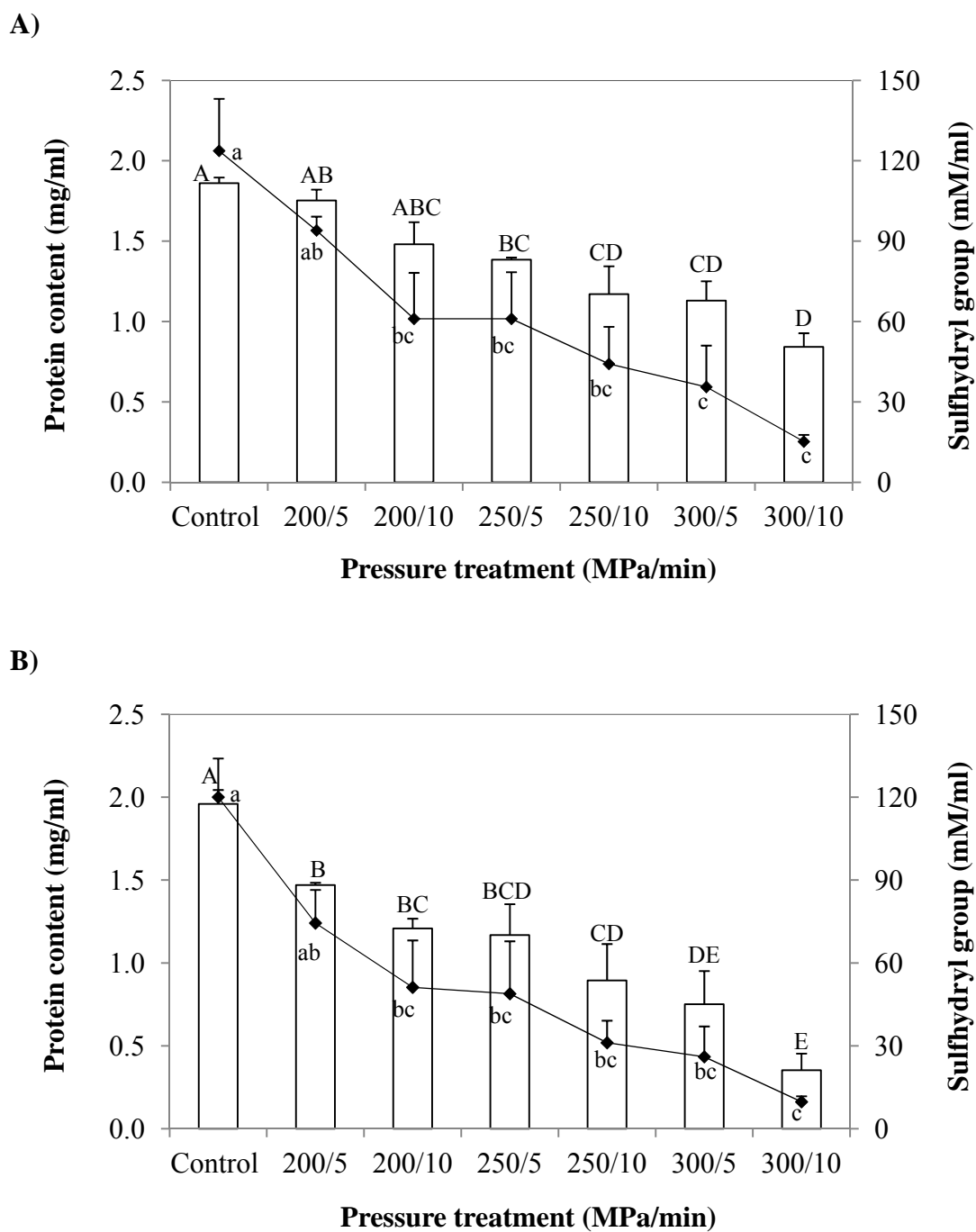 to 1.47 mg/ml (decreased by 25%) was observed for strain 10292 after a treatment of 200 MPa for 5 min (Fig. 5.3B). The protein contents of strains 10290 and 10292 significantly decreased to 1.17 mg/ml (decreased by 37%) and 0.89 mg/ml (decreased by 54%), respectively, after a treatment of 250 MPa for 10 min and further decreased to 0.84 mg/ml (decreased by 55%) and 0.35 mg/ml (decreased by 82%), respectively, after a treatment of 300 MPa for 10 min. A similar trend in decreases of total SH groups in the cellular extracts of both *V. parahaemolyticus* strains as the pressure of a treatment increased was also observed. SH contents of strains 10290 and 10292 significantly decreased from 124 to 61 mM/ml (decreased by 51%) and from 120 to 51 mM/ml (decreased by 57%), respectively, after treatment of 200 MPa for 10 min and further decreased to 15 mM/ml (decreased by 88%) and 10 mM/ml (decreased by 92%), respectively, after a treatment of 300 MPa for 10 min.

Protein denaturation has been proposed as a possible mechanism for pressure inactivation of microorganisms (Hayakawa and others 1994). Under pressures, protein denaturation may occur through weak hydrophobic and electrostatic interactions, resulting in structural changes and loss of functionality (Grigera and McCarthy 2010; Balny and Mason 1993). Once protein is partially unfolded under a pressure treatment, SH groups become oxidized and form disulfide bonds to stabilize protein structure. The decrease in SH contents observed in pressurized *V. parahaemolyticus* cells (Fig. 5.3) indicates that protein aggregation occurred under pressures as a result of protein denaturation. Hayman and others (2008) investigated effects of pressure on aggregation of lactate dehydrogenase (LDH) of 122 kDa in phosphate buffer (pH 7.4) using dynamic light scattering and observed two molecules (104 and 68,488 kDa) originating from LDH after a pressure treatment of 600 MPa for 5 min. These observations indicate that enzymes and proteins in bacterial cells may be denatured or aggregated following pressure treatments, leading to cell death.

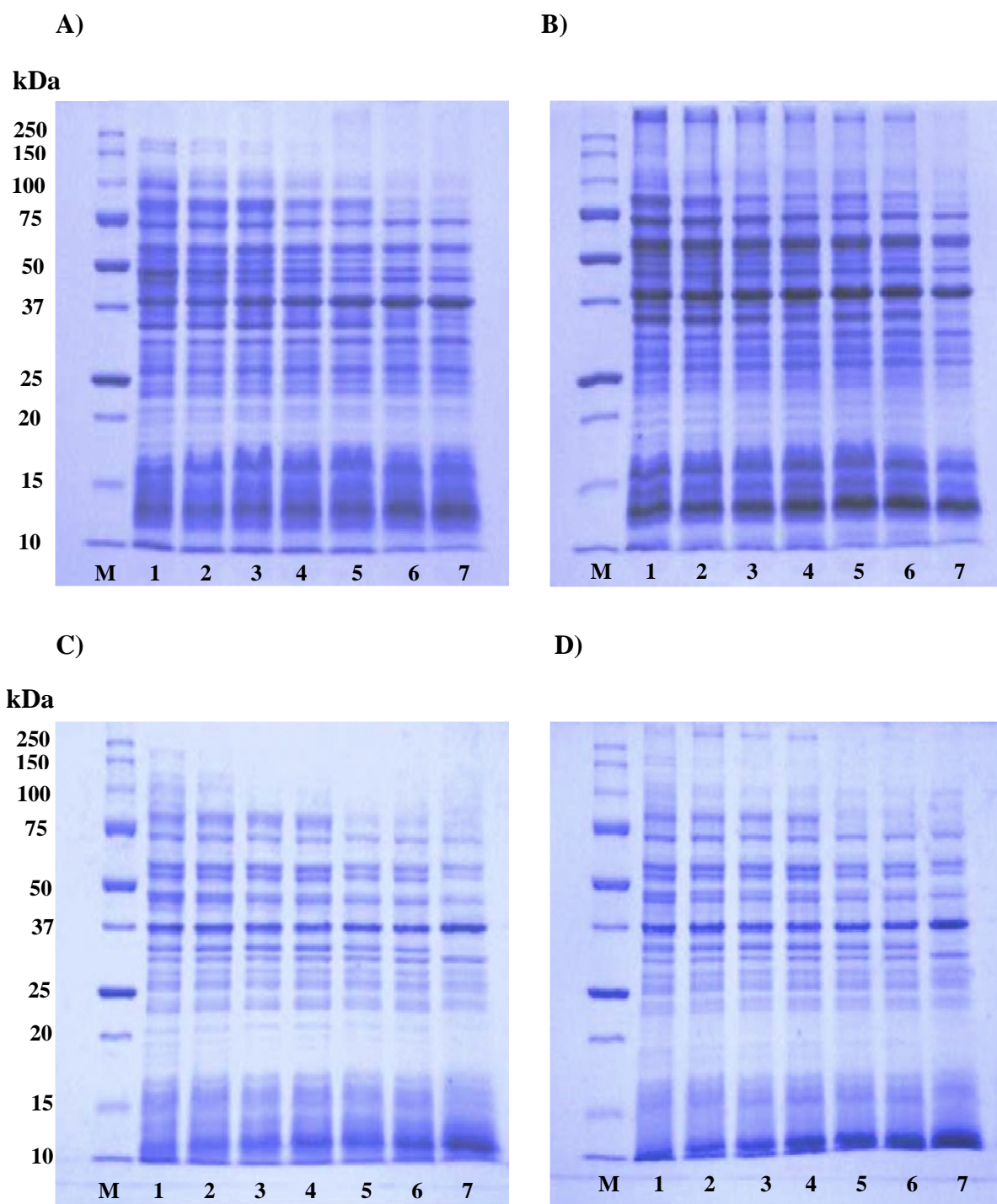Fig 5.4 SDS-PAGE analysis of proteins extracted from cells of *Vibrio parahaemolyticus* 10290 (10 μg) under reducing (A) and non-reducing (B) conditions and 10292 (5 μg) under reducing (C) and non-reducing (D) conditions. Lane 1; no pressure treatment, Lane 2: 200 MPa for 5 min, Lane 3: 200 MPa for 10 min, Lane 4: 250 MPa for 5 min, Lane 5: 250 MPa for 10 min, Lane 6: 300 MPa for 5 min and Lane 7: 300 MPa for 10 min.

5.4.4 Analysis of cellular proteins by SDS-PAGE

Profiles of cellular proteins extracted from cells of *V. parahaemolyticus* 10290 and 10292 with or without pressure treatments were analyzed by SDS-PAGE (Fig. 5.4). Two protein bands with molecular weights between 150 and 250 kDa observed for untreated 10290 (Fig. 5.4A-B, Lane 1) disappeared as pressure treatments increased (Fig. 5.4A-B, Lane 2-7) regardless of running gels under reducing or non-reducing condition. In addition, the intensity of certain protein bands with molecular weights between 75 and 100 kDa also decreased as the pressure of treatments increased. Similar changes in cellular protein profiles after pressure treatments were observed for strain 10292 (Fig. 5.4C-D). Furthermore, an increase in the intensity of a protein band (about 37 kDa) with increased pressure treatments was observed for both strains. These observations demonstrate that protein profiles of *V. parahaemolyticus* cells can be altered under pressure treatments. Wang and others (2013) also reported a loss of two large protein fragments (130 and 140 kDa) and an increase in intensity of a 40 kDa fragment in cellular proteins of *V. parahaemolyticus* after treatments of 200 to 300 MPa for 10 min. A study of *S.* Typhimurium reported a significant alteration of outer membrane proteins (Omp) with a remaining intensity of a 35-kDa protein (OmpA) after a treatment of 600 MPa for 10 min (Ritz and others 2000). The OmpA is known to associate with shape and integrity of bacterial cells and very resistant to stresses (Nikaido and Vaara 1985). In this study, the protein fragment in cellular protein profiles of both *V. parahaemolyticus* strains 10290 and 10292 with a molecular weight of 37 kDa that was pressure-resistant might be associated with outer membrane proteins.

5.4.5 Analysis of protease activity in cellular proteins extracted by B-PER reagent

It was hypothesized that proteases in *V. parahaemolyticus* cells would be released from ruptured cells after pressure treatments, resulting in breaking down protein molecules extracted from the cell pellet. Analysis of cellular protein extracts obtained from both *V. parahaemolyticus* strains after pressure treatments (200, 250 and 300 MPa for 10 min) revealed a very low protease activity (70 - 150 ng/ml for 10290 and 50 - 100

ng/ml for 10292) which were similar to those detected in cellular protein extracts from cells without pressure treatments. In addition, no detectable protease activity (<50 ng/ml) was observed in the 2% NaCl solution and the B-PER extraction reagent used for protein extraction. These data indicate that protease activity was not involved in the changes of cellular protein profiles, particularly the loss of certain protein bands on the SDS-PAGE gels, of *V. parahaemolyticus* cells observed in this study. It is highly likely that proteases were either denatured or altered to lose its function after the pressure treatments. It was reported that protease activities in cells of *Saccharomyces cerevisiae* and *Escherichia coli* were completely inactivated after a treatment at 448 MPa for 30 s (Bang and Chung 2010).

5.4.6 Analysis of proteins extracted with urea from cell pellet

SDS-PAGE analyses of proteins extracted with 4M urea from cell pellet of pressure-treated *V. parahaemolyticus* strains 10290 and 10292 after the protein extraction with B-PER protein extraction reagent are reported in Fig. 5.5. The proteins (molecular weights of 150 - 250 kDa and 75 - 100 kDa) which disappeared from the profiles of proteins extracted with the B-PER protein extraction reagent after pressure treatments (Fig. 5.5A and 5.5C) were captured in the profiles of proteins extracted with 4M urea (Fig. 5.5B and 5.5D). The recovery of these protein fragments in the cell pellet of both *V. parahaemolyticus* strains 10290 and 10292 after the pressure treatments provided further evidence that protein aggregation occurred during the treatments.

In conclusions, inactivation of *V. parahaemolyticus* cells by high pressure processing is associated with cell damage and changes of cellular protein profiles. The major change of the cellular proteins under pressures is a decrease in total SH contents induced by pressures. The pressure resistance of *V. parahaemolyticus* cells varies among strains. Therefore, effects of high pressure processing on alterations of cell morphology and proteins profiles of *V. parahaemolyticus* depend on the bacteria strains and pressure applied.
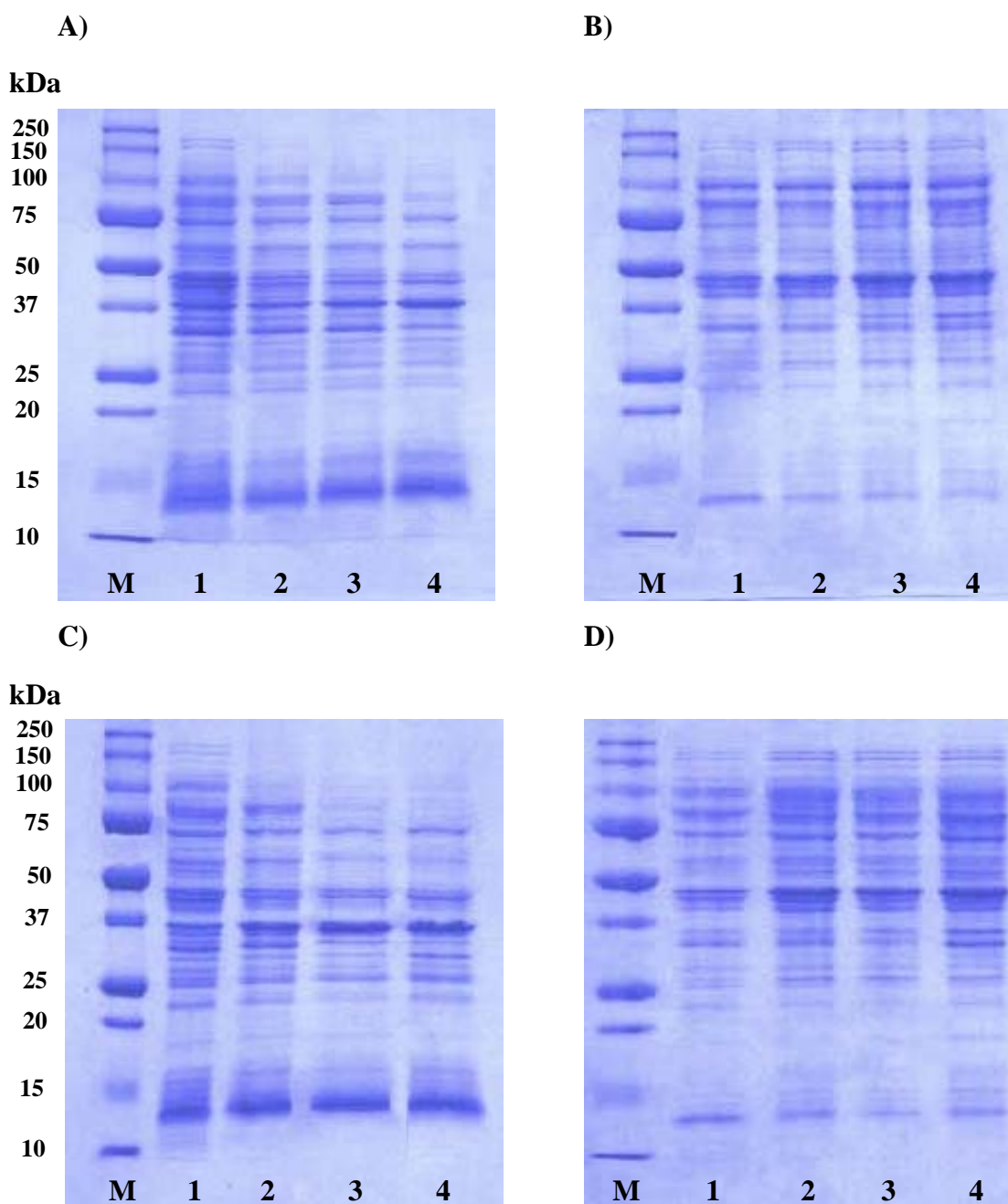
Fig 5.5 SDS-PAGE analysis of proteins in cell pellet of *Vibrio parahaemolyticus* extracted with B-PER protein extraction reagent and 4M urea after pressure treatments under reducing condition. Strain 10290 extracted with B-PER reagent (A) and with 4M urea (B). Strain 10292 extracted with B-PER reagent (C) and with 4 M urea (D). Lane 1: no pressure treatment, Lane 2: 200 MPa, 10 min, Lane 3: 250 MPa, 10 min and Lane 4: 300 MPa for 10 min.

5.5 Acknowledgements

CHAPTER 6

GENERAL CONCLUSIONS

The aim of this project was to develop effective treatments for reducing *V. parahaemolyticus* contamination in oysters, which would decrease risks of *V. parahaemolyticus* infections associated with raw oyster consumption. A total of four studies were conducted to develop low-temperature depuration and low-temperature high hydrostatic pressure processing (HPP) as post-harvest treatments for raw oysters.

The first study investigating temperature effects on depuration for reducing *V. parahaemolyticus* contamination in oysters revealed that depuration using refrigerated seawater (7 and 15 ºC) enhanced the efficacy of the process in reducing *V. parahaemolyticus* in oysters without apparent negative effects. Depuration of oysters with UV-sterilized artificial seawater (30 ppt) at temperatures ranging from 7 to 15 ºC for 5 days resulted in >3.0 log MPN/g reductions of five clinical *V. parahaemolyticus* strains in oysters.

In the second study, factors, including water salinity and types (diploid and triploid) and sizes (extra-small and medium) of oysters were examined. Oysters exhibited biological activity of oysters in water with a salinity between 20 and 30 ppt. Greater reductions (>3.0 log MPN/g) of *V. parahaemolyticus* were observed in oysters when depurated in artificial seawater at 12.5 ºC for 5 days with a salinity of 20 or 30 ppt than that (2.1 log MPN/g) in water with a lower salinity of 10 ppt. Reduction of *V. parahaemolyticus* in oysters by depuration was not influenced by types and sizes of oysters. Water temperature and water salinity are important factors affecting efficacy of depuration for decontaminating *V. parahaemolyticus* in oysters. Depuration of oysters in seawater with a salinity of 25 ppt at 12.5 ºC for 5 days applied as a post-harvest process for reducing *V. parahaemolyticus* in contaminated oysters.

The third study investigating variation in pressure resistance among clinical and environmental *V. parahaemolyticus* strains as well as effects of low temperatures (15, 5

and 1.5 ºC) on pressure treatments (200 to 300 MPa for 5 and 10 min) for inactivating *V. parahaemolyticus* cells. This study demonstrated variation in pressure resistance among clinical and environmental *V. parahaemolyticus* strains. The efficacy of high hydrostatic pressure processing (HPP) in inactivating *V. parahaemolyticus* cells in culture suspension or oyster homogenates was greatly enhanced by lowering the processing temperature from 20 to 1.5 °C. A HPP of 250 MPa at 1.5°C for 5 min completely inactivated all *V. parahaemolyticus* strains (>6.4 log CFU/g) in oyster homogenates. This low-temperature HPP could be adapted by the shellfish industry to produce fresh-like raw oysters which are free of *V. parahaemolyticus* for safe consumption.

The last study examined the mechanism of HPP for inactivating *V. parahaemolyticus* cells. HPP caused cell membrane damage as indicated by detection of intracellular materials in supernatants of *V. parahaemolyticus* culture suspension after pressure treatments. Scanning electron microscopy images clearly revealed broken cells of *V. parahaemolyticus* after a treatment of 300 MPa for 5 min. In addition, protein denaturation and changes in protein profiles of cellular proteins were also observed from pressurized *V. parahaemolyticus* cells. A proposed mechanism of HPP for inactivating *V. parahaemolyticus* is that HPP causes cell membrane damage, denaturation of cellular proteins and aggregation of cellular proteins. Understanding the mechanism of *V. parahaemolyticus* inactivation by HPP would allow development of optimized HPP for processing raw oysters for safe consumption.

In conclusion, this project developed two new processes (low-temperature depuration and low-temperature HPP) which could be applied as post-harvest treatments of oysters to minimize *V. parahaemolyticus* infections associated with consumption of raw oysters. Future studies are needed to validate the two processes for reducing *V. parahaemolyticus* in contaminated oysters at a commercial scale for industrial application as post-harvest processes.

BIBLIOGRAPHY

[BAM] Bacteriological Analytical Manual. 2004. Chapter 9: Vibrio. Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm. Accessed on May 5, 2014.

[CDC] Centers for Disease Control and Prevention. 1993. *Vibrio vulnificus* infections associated with raw oyster consumption -- Florida, 1981-1992. Morb Mortal Wkly Rep 42:405-7.

[CDC] Centers for Disease Control and Prevention. 1996. *Vibrio vulnificus* infections associated with eating raw oysters -- Los Angeles, 1996. Morb Mortal Wkly Rep 45:621-4.

[CDC] Centers for Disease Control and Prevention. 1998. Outbreak of *Vibrio parahaemolyticus* infections associated with eating raw oysters -- Pacific Northwest, 1997. Morb Mortal Wkly Rep 47(22):457-62.

[CDC] Centers for Disease Control and Prevention. 1999. Outbreak of *Vibrio parahaemolyticus* infection associated with eating raw oysters and clams harvested from Long Island Sound -- Connecticut, New Jersey, and New York, 1998. Morb Mortal Wkly Rep 48:48-51.

[CDC] Centers for Disease Control and Prevention. 2005. *Vibrio* illnesses after Hurricane Katrina --- multiple states, August--September 2005. Morb Mortal Wkly Rep 54(37):928-31.

[CDC] Centers for Disease Control and Prevention. 2006. *Vibrio parahaemolyticus* infections associated with consumption of raw shellfish -- three states, 2006. Morb Mortal Wkly Rep 55(Dispatch):1-2.

[CDC] Centers for Disease Control and Prevention. 2013. Increase in *Vibrio parahaemolyticus* illnesses associated with consumption of shellfish from several Atlantic coast harvest areas, United States, 2013. Available at http://www.cdc.gov/vibrio/investigations/ Accessed on May 17, 2014.

[FAO/WHO] Food and Agriculture Organization/World Health Organization. 2005. Risk assessment of *Vibrio vulnificus* in raw oysters: interpretative summary and technical report. Microbiological risk assessment series no. 8. Rome: Food and Agriculture Organization.

[FDA] Food and Drug Administration. 2005. Quantitative risk assessment on the public health impact of pathogenic *Vibrio parahaemolyticus* in raw oysters. Available at: http://www.fda.gov/Food/FoodScienceResearch/RiskSafetyAssessment/ucm050421.htm Accessed on May 18, 2014.

[FDA] Food and Drug Administration. 2011. Fish and fisheries products hazards and control guidance. Available at: http://www.fda.gov/downloads/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Seafood/UCM251970.pdf. Accessed on May 18, 2014.

[FDA] Food and Drug Administration. 2012. Foodborne pathogenic microorganisms and natural toxins. Bad Bug Book. 2nd Edition. Available at http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/default.htm Acessed on May 18, 2014.

[FDA] Food and Drug Administration. 2013. CFR - code of federal regulations title 21. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?cfrpart=179&showfr=1. Accessed on May 3, 2014.

[NSSP] National Shellfish Sanitation Program. 2011. Guide for the control of molluscan shellfish. Available at: http://www.fda.gov/Food/GuidanceRegulation/FederalStateFoodPrograms/ucm2006754.htm Acessed on May 2, 2014.

[WHO] World Health Organization. 1999. *Vibrio parahaemolyticus*, Japan, 1996-1998. Wkly Epidemiol Rec 74(43):361-3.

Abad FX, Pintó RM, Gajardo R, Bosch A. 1997. Viruses in mussels: public health implications and depuration. J Food Prot 60:677-81.

Abbott SL, Powers C, Kaysner CA, Takeda Y, Ishibashi M, Joseph SW, Janda JM. 1989. Emergence of a restricted bioserovar of *Vibrio parahaemolyticus* as the predominant cause of *Vibrio*-associated gastroenteritis on the West coast of the United States and Mexico. J Clin Microbiol 27(12):2891-3.

Acres J, Gray J. 1978. Paralytic shellfish poisoning. Can Med Assoc J 18:1195-7.

Alam MJ, Miyoshi S, Shinoda S. 2003. Studies on pathogenic *Vibrio parahaemolyticus* during a warm weather season in the Seto Inland Sea, Japan. Environ Microbiol 5(8):706-10.

Allen K. 1899. The transmission of typhoid by sewage polluted oysters. Public Health Pap Rep 25:154-65.

Alpas H, Kalchayanand N, Bozoglu F, Sikes A, Dunne CP, Ray B. 1999. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. Appl Environ Microbiol 65(9):4248-51.

Altekruse SF, Bishop RD, Baldy LM, Thompson SG, Wilson SA, Ray BJ, Griffin PM. 2000. Vibrio gastroenteritis in the US Gulf of Mexico region: the role of raw oysters. Epidemiol Infect 124:489-95.

Andrews L, Jahncke M, Mallikarjunan K. 2003. Low dose gamma irradiation to reduce pathogenic *Vibrios* in live oysters (*Crassostrea virginica*). J Aquat Food Prod Technol 12:71-82.

Andrews LS. 2004. Strategies to control Vibrios in molluscan shellfish. Food Prot Trends 24:70-6.

Andrews LS, Park DL, Chen Y. 2000. Low temperature pasteurization to reduce the risk of *Vibrio* infections from raw shell-stock oysters. Food Addit Contam 19(7):787-91.

Ansaruzzaman M, Lucas M, Deen JL, Bhuiyan NA, Wang XY, Safa A, Sultana M, Chowdhury A, Nair GB, Sack DA, von Seidlein L, Puri MK, Ali M, Chaignat CL, Clemens JD, Barreto A. 2005. Pandemic serovars (O3:K6 and O4:K68) of *Vibrio parahaemolyticus* associated with diarrhea in Mozambique: spread of the pandemic into the African continent. J Clin Microbiol 43(6):2559-62.

Arcisz W, Kelly CB. 1955. Self-purification of the soft clam *Mya arenaria*. Public Health Rep 70(6):605-14.

Arroyo G, Sanz PD, Préstamo G. 1997. Effect of high pressure on the reduction of microbial populations in vegetables. J Appl Microbiol 82:735-42.

Ayres PA, Barrow GI. 1978. The distribution of *Vibrio parahaemolyticus* in British coastal waters: report of a collaborative study 1975-1976. J Hyg 80:281-94.

Baffone W, Citterio B, Vittoria E, Casaroli A, Pianetti A, Campana R, Bruscolini F. 2001. Determination of several potential virulence factors in *Vibrio* spp. isolated from seawater. Food Microbiol 18:479-88.

Bajovic B, Bolumar T, Heinz V. 2012. Quality considerations with high pressure processing of fresh and value added meat products. Meat Sci 92:280-9.

Balny C, Mason P. 1993. Effects of high-pressure on proteins. Food Rev Int 9:611-28.

Bang W, Chung H. 2010. Effect of high hydrostatic pressure on the enzyme activities in *Saccharomyces cerevisiae* and *Escherichia coli*. N Biotechnol 27:440-4.

Baross J, Liston J. 1970. Occurrence of *Vibrio parahaemolyticus* and related hemolytic vibrios in marine environments of Washington State. Appl Microbiol 20(2):179-86.

Beatrice G, Patrick S, Patrick M, Philippe G, Olivier LM, Tristan R. 2006. Diploid and triploid Pacific oysters, *Crassostrea gigas* (Thunberg), reared at two heights above sediment in Marennes-Oleron Basin, France: Difference in mortality, sexual maturation and hemocyte parameters. Aquaculture 254(1-4):606-16.

Bej AK, Patterson DP, Brasher CW, Vickery MCL, Jones DD, Kaysner CA. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. J Microbiol Methods 36:215-25.

Benito A, Ventoura G, Casadei M, Robinson T, Mackey B. 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. Appl Environ Microbiol 65:1564-9.

Beril C, Crance JM, Leguyader F, Apaire-Marchais V, Leveque F, Albert M, Goraguer MA, Schwartzbrod L, Billaudel S. 1996. Study of viral and bacterial indicators in cockles and mussels. Mar Pollut Bull 32(5):404-9.

Berlin DL, Herson DS, Hicks DT, Hoover DG. 1999. Response of pathogenic *Vibrio* species to high hydrostatic pressure. Appl Environ Microbiol 65(6):2776-80.

Blackstone GM, Nordstrom JL, Vickery MC, Bowen MD, Meyer RF, DePaola A. 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. J Microbiol Methods:149-55.

Blake NJ, Rodrick GE, Tamplin M, Luth P. 1985. Uptake and fate of bacteria in a laboratory depuration system. Proceedings of the tenth annual tropical and subtropical fisheries technological conference of the Americas. Florida Sea Grant. p. 227-48.

Blogoslawski WJ, Stewart ME. 1983. Depuration and public health. J World Maric Soc 14:535-45.

Blumer M, Souza G, Sass J. 1970. Hydrocarbon pollution of edible shellfish by an oil spill. Marine Biology 5:195-202.

Boschi-Pinto C, Lanata CF, Mendoza W, Habte D. 2006. Diarrheal diseases. In: Jamison DT, Feachem RG, Makgoba MW, Bos ER, Baingana FK, Hofman KJ, Rogo KO,

editors. Disease and mortality in Sub-Saharan Africa. 2 ed. Washington DC: Oxford University Press for the World Bank.

Bradford MM. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-54.

Bubb HD. 1975. *Vibrio parahaemolyticus* -- a marine pathogen detected in South African coastal waters. S Afr Med J 49(37):1514-6.

Buisson DH, Fletcher GC, Begg CW. 1981. Bacterial depuration of the Pacific oyster (*Crassostrea gigas*) in New Zealand. New Zeal J Sci 24:253-62.

Bull MK, Zerdin K, Howe E, Goicoechea D, Paramanandhan P, Stockman R, Sellahewa J, Szabo EA, Johnson RL, Stewart CM. 2004. The effect of high pressure processing on the microbial, physical and chemical properties of Valencia and Navel orange juice. Innov Food Sci Emerg Technol 5:135-49.

Butt AA, Aldridge KE, Sanders CV. 2004. Infections related to the ingestion of seafood. Part I: viral and bacterial infections. Lancet Infect Dis 4:201-12.

Cabello FC, Espejo RT, Hernandez MC, Rioseco ML, Ulloa J, Vergara JA. 2007. *Vibrio parahaemolyticus* O3:K6 epidemic diarrhea, Chile, 2005. Emerg Infect Dis 13(4):655-6.

Cai T, Jiang L, Yang C, Huang K. 2006. Application of real-time PCR for quantitative detection of *Vibrio parahaemolyticus* from seafood in eastern China. FEMS Immunol Med Microbiol 46:180-6.

Calia FM, Johnson DE. 1975. Bacteremia in suckling rabbits after oral challenge with *Vibrio parahaemolyticus*. Infect Immun 11(6):1222-5.

Calik H, Morrissey MT, Reno PW, An H. 2002. Effect of high-pressure processing on *Vibrio parahaemolyticus* strains in pure cultures and Pacific oysters. J Food Sci 67(4):1506-10.

Cann DC, Taylor LY, Merican Z. 1981. A study of the incidence of *Vibrio parahaemolyticus* in Malaysian shrimp undergoing processing for export. J Hyg 87:485-91.

Centre for Environment Fisheries and Aquaculture Science. 2010. CEFAS Protocol for inspection and approval of purification (depuration) systems - England and Wales. Available at http://www.cefas.defra.gov.uk/media/540968/purification generic protocol_v8 final.pdf Accessed on May 20, 2014.

Chae MJ, Cheney D, Su Y. 2009. Temperature effects on the depuration of *Vibrio parahaemolyticus* and *Vibrio vulnificus* from the American oyster (*Crassostrea virginica*). J Food Sci 74(2):M62-5.

Chatterjee BD, Sen T. 1974. *Vibrio parahaemolyticus* serotypes in Calcutta, India. Bull World Health Organ 50(6):559-61.

Chen Y, Liu X, Yan J, Li X, Mei L, Mao Q, Ma Y. 2010. Foodborne pathogens in retail oysters in south China. Biomed Environ Sci 23:32-6.

Chiou C, Hsu S, Chiu S, Wang T, Chao C. 2000. *Vibrio parahaemolyticus* serovar O3:K6 as cause of unusually high incidence of food-borne disease outbreaks in Taiwan from 1996 to 1999. J Clin Microbiol 38(12):4621-5.

Chong G, Cossius AR. 1983. A differential polarized phase fluorometric study of the effects of high hydrostatic pressure upon the fluidity of cellular membranes. Biochemistry 22:409-15.

Chowdhury MAR, Yamanaka H, Miyoshi S-I, Shinoda S. 1990. Ecology and Seasonal distribution of *Vibrio parahaemolyticus* in aquatic environments of a temperate region. FEMS Microbiol Ecol 74:1-9.

Considine KM, Kelly AL, Fitzgerald GF, Hill C, Sleator RD. 2008. High-pressure processing - effects on microbial food safety and food quality. FEMS Microbiol Lett 281:1-9.

Cook DW. 2003. Sensitivity of *Vibrio* species in phosphate-buffered saline and in oysters to high-pressure processing. J Food Prot 66(12):2276-82.

Cook DW, Bowers JC, DePaola A. 2002a. Density of total and pathogenic (*tdh*+) *Vibrio parahaemolyticus* in Atlantic and Gulf coast molluscan shellfish at harvest. J Food Prot 65(12):1873-80.

Cook DW, O'Leary P, Hunsucker JC, Sloan EM, Bowers JC, Blodgett RJ, DePaola A. 2002b. *Vibrio Vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: a national survey from June 1998 to July 1999. J Food Prot 65:79-87.

Cook DW, Ruple AD. 1989. Indicators bacteria and *Vibrionaceae* multiplication in post-harvest shellstock oysters. J Food Prot 52:343-9.

Cook DW, Ruple AD. 1992. Cold storage and mild heat treatment as processing aids to reduce the numbers of *Vibrio vulnificus* in raw oysters. J Food Prot 55(12):985-9.

Cordova JL, Astorga J, Silva W, Riquelme C. 2002. Characterization by PCR of *Vibrio parahaemolyticus* isolates collected during the 1997-1998 Chilean outbreak. Biol Res 35(3-4):433-40.

Costa Sobrinho PS, Destro MT, Franco BDGM, Landgraf M. 2010. Correlation between environmental factors and prevalence of *Vibrio parahaemolyticus* in oysters harvested in the southern coastal area of Sao Paulo State, Brazil. Appl Environ Microbiol 76(4):1290-3.

Costa Sobrinho PS, Destro MT, Franco BDGM, Landgraf M. 2011. Occurrence and distribution of *Vibrio parahaemolyticus* in retail oysters in Sao Paulo State, Brazil. Food Microbiol 28:137-40.

Croci L, Suffredini E, Cozzi L, Toti L. 2002. Effects of depuration of molluscs experimentally contaminated wtih *Escherichia coli*, *Vibrio cholerae* O1 and *Vibrio parahaemolyticus*. J Appl Microbiol 92:460-5.

Cruz-Romero M, Smiddy M, Hill C, Kerry JP, Kelly AL. 2004. Effects of high pressure treatment on physicochemical characteristics of fresh oysters (*Crassostrea gigas*). Innov Food Sci Emerg Technol 5:161-9.

Daniels NA, MacKinnon L, Bishop R, Altekruse S, Ray B, Hammond RM, Thompson S, Wilson S, Bean NH, Griffin PM, Slutsker L. 2000a. *Vibrio parahaemolyticus* infections in the United States, 1973-1998. J Infect Dis 181:1661-6.

Daniels NA, Ray B, Easton A, Marano N, Kahn E, McShan AL, Del Rosario L, Baldwin T, Kingsley MA, Puhr ND, Wells JG, Angulo FJ. 2000b. Emergence of a new *Vibrio parahaemolyticus* serotype in raw oysters: A prevention quandary. J Am Med Assoc 284(12):1541-5.

Deepanjali A, Kumar HS, Karunasagar I, Karunasagar I. 2005. Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the southwest coast of India. Appl Environ Microbiol 71:3575-80.

DePaola A, Hopkins LH, Peeler JT, Wentz B, McPhearson RM. 1990. Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. Appl Environ Microbiol 56(8):2299-302.

DePaola A, Jones JL, Noe KE, Byars RH, Bowers JC. 2009. Survey of postharvest-processed oysters in the United States for levels of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. J Food Prot 72(10):2110-3.

DePaola A, Jones JL, Woods J, Burkhardt W, III., Calci KR, Krantz JA, Bowers JC, Kasturi K, Byars RH, Jacobs E, Williams-Hill D, Nabe K. 2010. Bacterial and viral pathogens in live oysters: 2007 United States market survey. Appl Environ Microbiol 76(9):2754-68.

DePaola A, Kaysner CA, Bowers J, Cook DW. 2000. Environmental investigations of *Vibrio parahaemolyticus i*n oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). Appl Environ Microbiol 66:4649-54.

Drake SL, DePaola A, Jaykus L. 2007. An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Compr Rev Food Sci F 6:120-44.

Duan J, Su Y. 2005. Occurrence of *Vibrio parahaemolyticus* in two Oregon oyster growing bays. J Food Sci 70:M58-63.

Durães-Carvalho R, Souza AR, Martins LM, Sprogis AC, Bispo JA, Bonafe CF, Yano T. 2012. Effect of high hydrostatic pressure on *Aeromonas hydrophila* AH 191 growth in milk. J Food Sci 77:M417-24.

Eisenberg M, Topping JJ. 1984. Trace metal residues in shellfish from Maryland waters, 1976-1980. J Environ Sci B19:649-71.

European Communities. 1991. Council Directive 91/492 of 15 July 1991 laying down the health conditions for the production and the placing on the market of live bivalve molluscs. Official Journal of the European Communities L268:1-14.

Eyles MJ, Davey GR. 1984. Microbiology of commercial depuration of Sydney rock oyster, *Crassostrea commercialis*. J Food Prot 47:703-6.

Fang ZQ, Cheung RYH, Wong MH. 2003. Heavy metals in oysters, mussels and clams collected from coastal sites along the Pearl River Delta, South China. J Environ Sci 15:9-24.

Faruque SM, Albert MJ, Mekalanos JJ. 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol R 62:1301-14.

Fayer R, Graczyk TK, Lewis EJ, Trout JM, Farley CA. 1998. Survival of infectious *Cryptosporidium parvum* oocysts in seawater and Eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl Environ Microbiol 64:1070-4.

Fuenzalida L, Armijo L, Zabala B, Hernández C, Rioseco ML, Riquelme C, Espejo RT. 2007. *Vibrio parahaemolyticus* strains isolated during investigation of the summer 2006 seafood related diarrhea outbreaks in two regions of Chile. Int J Food Microbiol 117:270-5.

Fuenzalida L, Hernández C, Toro J, Rioseco ML, Romero J, Espejo RT. 2006. *Vibrio parahaemolyticus* in shellfish and clinical samples during two large epidemics of diarrhoea in southern Chile. Environ Microbiol 8(4):675-83.

Fujino T, Okuno Y, Nakada D, Aoyama A, Fukai K, Mukai T, Ueho T. 1953. On the bacteriological examination of shirasu-food poisoning. Med J Osaka Univ 4:299-304.

Fyfe M, Yeung ST, Daly P, Schallie K, Kelly MT, Buchanan S. 1997. Outbreak of *Vibrio parahaemolyticus* related to raw oysters in British Columbia. Can Commun Dis Rep 23:145-8.

García K, Torres R, Uribe P, Hernández C, Rioseco ML, Romero J, Espejo RT. 2009. Dynamics of clinical and environmental *Vibrio parahaemolyticus* strains during seafood-related summer diarrhea outbreaks in Southern Chile. Appl Environ Microbiol 75(23):7482-7.

Gil AI, Miranda H, Lanata CF, Prada A, Hall ER, Barreno CM, Nusrin S, Bhuiyan NA, Sack DA, Nair GB. 2007. O3:K6 serotype of *Vibrio parahaemolyticus* identical to the global pandemic clone associated with diarrhea in Peru. Int J Infect Dis 11(4):324-8.

González-Escalona N, Cachicas V, Acevedo C, Rioseco ML, Vergara JA, Cabello F, Romero J, Espejo RT. 2005. *Vibrio parahaemolyticus* diarrhea, Chile, 1998 and 2004. Emerg Infect Dis 11(1):129-31.

Gooch JA, DePaola A, Bowers J, Marshall DL. 2002. Growth and survival of *Vibrio parahaemolyticus* in postharvest American oysters. J Food Prot 65(6):970-4.

Gooch JA, DePaola A, Kaysner CA, Marshall DL. 2001. Evaluation of two direct plating methods using nonradioactive probes for enumeration of *Vibrio parahaemolyticus* in oysters. Appl Environ Microbiol 67:721-4.

Gould GW. 2000. Preservation: past, present and future. Br Med Bull 56(1):85-96.

Greenberg EP, Duboise M, Palhof B. 1982. The survival of marine vibrios in *Mercenaria mercenaria*, the hardshell clam. J Food Safety 4:113-23.

Grigera JR, McCarthy AN. 2010. The behavior of the hydrophobic effect under pressure and protein denaturation. Biophys J 98:1626–31.

Hackney CR, Kleeman EG, Ray B, Speck ML. 1980. Adherence as a method of differentiating virulent and avirulent strains of *Vibrio parahaemolyticus*. Appl Environ Microbiol 40(3):652-8.

Hamada M, Ishizaki S, Nagai T. 1994. Variation of SH content and kamaboko-gel forming ability of shark muscle protein by electrolysis. J Shimonoseki Univ Fish 42:131-5.

Harding JM. 2007. Comparison of growth rates between diploid DEBY eastern oysters (*Crassostrea viginica*, Gmelin, 1971), triploid eastern oysters, and triploid Suminoe oysters (*C. ariakensis*, Fugita 1913). Journal of Shellfish Research 26(4):961-72.

Harth E, Matsuda L, Hernández C, Rioseco ML, Romero J, González-Escalona N, Martínez-Urtaza J, Espejo RT. 2009. Epidemiology of *Vibrio parahaemolyticus* outbreaks, Southern Chile. Emerg Infect Dis 15(2):163-8.

Hayakawa I, Kanno T, Tomita M, Fujio Y. 1994. Application of high pressure for spore inactivation and protein denaturation. J Food Sci 59:159-63.

Hayashi R, Kawamura Y, Nakasa T, Okinaka O. 1989. Application of high pressure to food processing: pressurization of egg white and yolk, and properties of gels formed. Agric Biol Chem 53(11):2935-9.

Hayman MM, Kouassi GK, Anantheswaran RC, Floros JD, Knabel SJ. 2008. Effect of water activity on inactivation of *Listeria monocytogenes* and lactate dehydrogenase during high pressure processing. Int J Food Microbiol 124:21-6.

He H, Adams RM, Farkas DF, Morrissey MT. 2002. Use of high-pressure processing for oyster shucking and shelf-life extension. J Food Sci 67(2):640-5.

Hervio-Heath D, Colwell RR, Derrien A, Robert-Pillot A, Fournier JM, Pommepuy M. 2002. Occurrence of pathogenic vibrios in coastal areas of France. J Appl Microbiol 92:1123-35.

Honda S, Matsumoto S, Miwatani T, Honda T. 1992. A survey of urease-positive *Vibrio parahaemolyticus* strains isolated from traveller's diarrhea, sea water and imported frozen sea foods. Eur J Epidemiol 8(6):861-4.

Honda T, Iida T. 1993. The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysins. Rev Med Microbiol 4(2):106-13.

Honda T, Ni Y, Miwatani TM. 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infect Immun 56(4):961-5.

Honda T, Taga S, Takeda T, Hasibuan MA, Takeda Y, Miwatani T. 1976. Identification of lethal toxin with the thermostable direct hemolysin produced by *Vibrio parahaemolyticus*, and some physicochemical properties of the purified toxin. Infect Immun 13(1):133-9.

Hood MA, Ness GE, Rodrick GE, Blake NJ. 1983. Effects of storage on microbial loads of two commercially important shellfish species, *Crassostrea virginica* and *Mercenaria campechiensis*. Appl Environ Microbiol 45:1121-8.

Hooper WL, Barrow GI, Mcnab DJN. 1974. *Vibrio parahaemolyticus* food-poisoning in Britain. Lancet 303(7866):1100-2.

Hoover DG, Metrick C, Papineau AM, Farkas DF, Knorr D. 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food Technol 43:99-107.

Huq A, Colwell RR. 1995. Vibrios in the marine and estuarine environment. J Mar Biotechnol 3:60-3.

Iida T, Park K-S, Suthienkul O, Kozawa J, Yamaichi Y, Yamamoto K, Honda T. 1998. Close proximity of the *tdh*, *trh* and *ure* genes on the chromosome of *Vibrio parahaemolyticus*. Microbiology 144:2517-23.

Iida T, Suthienkul O, Park KS, Tang GQ, Yamamoto RK, Ishibashi M, Yamamoto K, Honda T. 1997. Evidence for genetic linkage between the *ure* and *trh* genes in *Vibrio parahaemolyticus*. J Med Microbiol 46(8):639-45.

Isobe T, Takada H, Kanai M, Tsutsumi S, Isobe KO, Boonyatumanond R, Zakaria MP. 2007. Distribution of polycyclic aromatic hydrocarbons (PAHs) and phenolic

endocrine disrupting chemicals in South and Southeast Asian mussels. Environ Monit Assess 135:423-40.

Iwamoto M, Ayers T, Mahon BE, Swerdlow DL. 2010. Epidemiology of seafood-associated infections in the United States. Clin Microbiol Rev 23(2):399-411.

Jakabi M, Gelli DS, Torre JC, Rodas MA, Franco BD, Destro MT, Landgrafi M. 2003. Inactivation by ionizing radiation of *Salmonella enteritidis*, *Salmonella infantis*, and *Vibrio parahaemolyticus* in oysters (*Crassostrea brasiliana*). J Food Prot 66(6):1025-9.

Jiang X, Chai T. 1996. Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. Appl Environ Microbiol 62:1300-5.

Jiann K, Presley BJ. 1997. Variations in trace metal concentrations in American oysters (*Crassostrea virginica*) collected from Galveston Bay, Texas. Estuaries 20:710-24.

Johnson WG, Jr., Salinger AC, King WC. 1973. Survival of *Vibrio parahaemolyticus* in oyster shellstock at two different storage temperatures. Appl Microbiol 26:122-3.

Kalab M. 1970. Factors affecting the Ellman determination of sulfhydryl groups in skimmilk powder and gels. J Dairy Sci 53(6):711-8.

Kalchayanand N, Frethem C, Dunne P, Sikes A, Ray B. 2002. Hydrostatic pressure and bacteriocin-triggered cell wall lysis of *Leuconostoc mesenteroides*. Innov Food Sci Emerg Technol 3:33-40.

Kaletunç G, Lee J, Alpas H, Bozoglu F. 2004. Evaluation of structural changes Induced by high hydrostatic pressure in *Leuconostoc mesenteroides*. Appl Environ Microbiol 70:1116-22.

Kaneko T, Colwell RR. 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. J Bacteriol 113(1):24-32.

Kaper JB, Campen RK, Seidler RJ, Baldini MM, Falkow S. 1984. Cloning of the thermostable direct or Kanagawa phenomenon-associated hemolysin of *Vibrio parahaemolyticus*. Infect Immun 45(1):290-2.

Karunasagar I, Sugumar G, Karunasagar I, Reilly PJA. 1996. Rapid polymerase chain reaction method for detection of Kanagawa positive *Vibrio parahaemolyticus* in seafoods. Int J Food Microbiol 31:317-23.

Kaspar CW, Tamplin ML. 1993. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl Environ Microbiol 59(8):2425-9.

Kaysner CA, Tamplin M, Wekell MM, Stott RF, Colburn KG. 1989. Survival of *Vibrio vulnificus* in shellstock ans shucked oysters (*Crassostrea gigas* and *Crassostrea virginica*) and effects of isolation medium on recovery. Applied and Environmental Microbiology 55(12):3072-9.

Kelly MT, Dinuzzo A. 1985. Uptake and clearance of *Vibio vulnificus* from Gulf coast oysters (*Crassostrea virginica*). Appl Environ Microbiol 50(6):1548-9.

Kelly MT, Stroh EMD. 1988. Temporal relationship of *Vibrio parahaemolyticus* in patients and the environment. J Clin Microbiol 26(9):1754-6.

Kelly MT, Stroh EMD. 1989. Urease-positive, Kanagawa-negative *Vibrio parahaemolyticus* from patients and the environment in the Pacific Northwest. J Clin Microbiol 27(12):2820-2.

Kilgen MB, Hemard MT, Duet D, Rabalais S. 2001. Collaborative evaluation of commercial irradiation for *Vibrio vulnificus* control in Louisiana oysters. Louisiana Sea Grant Rep.

Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M. 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the toxR gene. J Clin Microbiol 37:1173-7.

Kirkland KB, Meriwether RA, Leiss JK, MacKenzie WR. 1996. Steaming oysters does not prevent Norwalk-like gastroenteritis. Public Health Rep 111:527-30.

Knorr D. 1999. Novel approaches in food-processing technology: new technologies for preserving foods and modifying function. Curr Opin Chem Biol 10:485-91.

Koo J, Jahncke ML, Reno PW, Hu X, Mallikarjunan P. 2006. Inactivation of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in phosphate-buffered saline and in inoculated whole oysters by high pressure processing. J Food Prot 69(3):596-601.

Kural AG, Chen H. 2008. Conditions for a 5-log reduction of *Vibrio vulnificus* in oysters through high hydrostatic pressure treatment. Int J Food Microbiol 122:180-7.

Kural AG, Shearer AEH, Kingsley DH, Chen H. 2008. Conditions for high pressure inactivation of *Vibrio parahaemolyticus* in oysters. Int J Food Microbiol 127:1-5.

La Peyre JF, Cooper RK, Tiersch TR. 2003. Elimination of *Vibrio vulnificus* in triploid Eastern oysters. Oyster research and Restoration in U.S. Coastal Waters: Strategies for the Future. Annapolis, Maryland (Abstract).

Laemmli U. 1970. Cleavage of structural proteins during assembly the head of bacteriophage T4. Nature 227:680-5.

Lam S, Yu M, Sng EH, Doraisingham S. 1974. First isolations of *Vibrio parahaemolyticus* in Singapore. Singapore Med J 15(3):184-7.

Leal NC, da Silva SC, Cavalcanti VO, Figueiroa AC, Nunes VV, Miralles IS, Hofer E. 2008. *Vibrio parahaemolyticus* serovar O3:K6 gastroenteritis in northeast Brazil. J Appl Microbiol 105(3):691-7.

Lee C, Pan S, Chen C. 1995. Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with the PCR. Appl Environ Microbiol 61:1311-7.

Lee J, Jung D, Eom S, Oh S, Kim Y, Kwak H, Kim Y. 2008. Occurrence of *Vibrio parahaemolyticus* in oysters from Korean retail outlets. Food Control 19:990-4.

Lemoine T, Germanetto P, Giraud P. 1999. Toxi-infection alimentaire collective a` *Vibrio parahaemolyticus*. Bull Epidemiol Hebd 10:37-8.

Levine WC, Griffin PM. 1993. Vibrio infections on the Gulf Coast: results of first year of regional surveillance. Gulf Coast Vibrio Working Group. J Infect Dis 167(2):479-83.

Lewis M, Rikard S, Arias CR. 2010. Evaluation of a flow-through depuration system to eliminate the human pathogen *Vibrio vulnificus* from oysters. Journal of Aquaculture Research & Development 1(2):1-6.

Lin Z, Kumagai K, Baba K, Mekalanos JJ, Nishibuchi M. 1993. *Vibrio parahaemolyticus* has a homolog of the *Vibrio cholerae toxRS* operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. J Bacteriol 175:3844-55.

Linton M, Mc Clements JMJ, Patterson MF. 2003. Changes in the microbiological quality of shellfish, brought about by treatment with high hydrostatic pressure. Int J Food Sci Technol 38:713-27.

Lipp EK, Farrah SA, Rose JB. 2001. Assessment and impact of microbial fecal pollution and human enteric pathogens in a coastal community. Mar Pollut Bull 42:286-93

Liu C, Lu J, Su Y. 2009. Effects of flash freezing followed by frozen storage on reducing *Vibrio parahaemolyticus* in Pacific raw oyster (*Crassostrea gigas*). J Food Prot 72:174-7.

Loosanoff VL. 1958. Some aspects of behavior of oysters at different temperatures. Biol Bull 114:57-70.

Lozano-León A, Torres J, Osorio CR, Martínez-urtaza J. 2003. Identification of tdh-positive *Vibrio parahaemolyticus* form an outbreak associated with raw oyster consumption in Spain. FEMS Microbiol Lett 226:281-4.

Ma L, Su Y. 2011. Validation of high pressure processing for inactivating *Vibrio parahaemolyticus* in Pacific oysters (*Crassostrea gigas*). Int J Food Microbiol 144:469-74.

Mahmoud BSM, Burrage DD. 2009. Inactivation of *Vibrio parahaemolyticus* in pure culture, whole live and half shell oysters (*Crassostrea virginica*) by X-ray. Lett Appl Microbiol 48:572-8.

Malone AS, Shellhammer TH, Courtney PD. 2002. Effects of high pressure on the viability, morphology, lysis, and cell wall hydrolase activity of *Lactococcus lactis* subsp. *cremoris*. Appl Environ Microbiol 68:4357-63.

Mañas P, Pagán R. 2005. Microbial inactivation by new technologies of food preservation. J Appl Microbiol 98:1387-99.

Martinez-Urtaza J, Simental L, Velasco D, DePaola A, Ishibashi M, Nakaguchi Y, Nishibuchi M, Carrera-Flores D, Rey-Alvarez C, Pousa A. 2005. Pandemic *Vibrio parahaemolyticus* O3:K6, Europe. Emerg Infect Dis 11(8):1319-20.

Matté GR, Matté MH, Rivera IG, Martins MT. 1994. Distribution of potentially pathogenic vibrios in oysters from a tropical region. J Food Prot 57(10):870-3.

McCarthy SA, DePaola A, Cook DW, Kaysner CA, Hill WE. 1999. Evaluation of alkaline phosphatase- and digoxigenin-labelled probes for detection of the thermolabile hemolysin (*tlh*) gene of *Vibrio parahaemolyticus*. Lett Appl Microbiol 28:66-70.

McLaughlin JB, DePaola A, Bopp CA, Martinek KA, Napolilli NP, Allison CG, Murray SL, Thompson EC, Bird MM, Middaugh JP. 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N Engl J Med 353:1463-70.

Melody K, Senevirathne R, Jane M, Jaykus LA, Supan J. 2008. Effectiveness of icing as a post harvest treatment for control of *Vibrio vulnificus* and *Vibrio*

parahaemolyticus in the Eastern oyster (*Crassostrea virginica*). J Food Prot 71(7):1475-80.

Miyamoto Y, Kato T, Obara Y, Akiyama S, Takizawa K, Yamai S. 1969. In vitro hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. J Bacteriol 100(2):1147-9.

Miyamoto Y, Obara Y, Nikkawa T, Yamai S, Kato T, Yamada Y, Ohashi M. 1980. Simplified purification and biophysicochemical characteristics of kanagawa phenomenon-associated hemolysin of *Vibrio parahaemolyticus*. Infect Immun 28(2):567-76.

Molenda JR, Johnson WG, Fishbein M, Wentz B, Mehlman IJ, Dadisman TA, Jr. 1972. *Vibrio parahaemolyticus* gastroenteritis in Maryland: Laboratory aspects. Appl Microbiol 24(3):444-8.

Molero X, Bartolome RM, Vinuesa T, Guarner L, Accarino A, Casellas F, Garcia R. 1989. Acute gastroenteritis due to *Vibrio parahaemolyticus* in Spain: Presentation of 8 cases. Med Clin-Barcelona 92:1-4.

Motes ML, DePaola A. 1996. Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*). Applied and Environmental Microbiology 62:3875-7.

Motes ML, DePaola A, Cook DW, Veazey JE, Hunsucker JC, Garthright WE, Blodgett RJ, Chirtel SJ. 1998. Influence of water temperature and salinity on *Vibrio vulnificus* in Northen Gulf and Atlatic coast oysters (*Crassostrea virginica*). Appl Environ Microbiol 64(4):1459-65.

Muntada-Garriga JM, Rodriguez-Jerez JJ, Lopez-Sabater EI, Mora-Ventura MT. 1995. Effect of chill and freezing temperatures on survival of *Vibrio parahaemolyticus* inoculated in homogenates of oyster meat. Lett Appl Microbiol 20(4):225-7.

Murchie LW, Cruz-Romero M, Kerry JP, Linton M, Patterson MF, Smiddy M, Kelly AL. 2005. High pressure processing of shellfish: A review of microbiological and other quality aspects. Innov Food Sci Emerg Technol 6:257-70.

Nell JA, Cox E, Smith IR, Maguire GB. 1994. Studies on triploid oysters in Australia. I.The farming potential of triploid Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley). Aquaculture 126(3-4):243-55.

Newton A, Kendall M, Vugia DJ, Henao OL, Mahon BE. 2012. Increasing rates of vibriosis in the United States, 1996-2010: review of surveillance data from 2 systems. Clin Infect Dis 54(S5):S391-5.

Nikaido H, Vaara M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol Rev 49:1-32.

Nishibuchi M, Ishibashi M, Takeda Y, Kaper JB. 1985. Detection of the thermostable direct hemolysin gene and related DNA sequence in *Vibrio parahaemolyticus* and other *Vibrio* species by the DNA colony hybridization test. Infect Immun 49(3):481-6.

Nishibuchi M, Kaper JB. 1985. Nucleotide sequence of the thermostable direct hemolysin gene of *Vibrio parahaemolyticus*. J Bacteriol 162(2):558-64.

Nishibuchi M, Kaper JB. 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infect Immun 63(6):2093-9.

Nolan CM, Ballard J, Kaysner CA, Lilja JL, Williams LP, Jr., Tenover FC. 1984. *Vibrio parahaemolyticus* gastroenteritis: An outbreak associated with raw oysters in the Pacific Northwest. Diagn Microbiol Infect Dis 2(2):119-28.

Nordstrom JL, Kaysner CA, Blackstone GM, Vickery MCL, Bowers JC, DePaola A. 2004. Effect of intertidal exposure on *Vibrio parahaemolyticus* levels in Pacific Northwest oysters. J Food Prot 67(10):2178-82.

Nordstrom JL, Vickery MCL, Blackstone GM, Murray SL, DePaola A. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl Environ Microbiol 73:5840-7.

Normanno G, Parisi A, Addante N, Quaglia NC, Dambrosio A, Montagna C, Chiocco D. 2006. *Vibrio parahaemolyticus*, *Vibrio vulnificus* and microorganisms of fecal origin in mussels (*Mytilus galloprovincialis*) sold in the Puglia region (Italy). Int J Food Microbiol 106(2):219-22.

Okuda J, Ishibashi M, Abbott SL, Janda JM, Nishibuchi M. 1997a. Analysis of the thermostable direct hemolysin (*tdh*) gene and the *tdh*-related hemolysin (*trh*) genes in urease-positive strains of *Vibrio parahaemolyticus* isolated on the West coast of the United States. J Clin Microbiol 35(8):1965-71.

Okuda J, Ishibashi M, Hayakawa E, Nishino T, Takeda Y, Mukhopadhyay AK, Garg S, Bhattacharya SK, Nair GB, Nishibuchi M. 1997b. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from southeast Asian travelers arriving in Japan. J Clin Microbiol 35(12):3150-5.

Osawa R, Okitsu T, Morozumi H, Yamai S. 1996. Occurrence of urease-positive *Vibrio parahaemolyticus* in Kanagawa, Japan, with specific reference to presence of thermostable direct hemolysin (TDH) and the TDH-related-hemolysin genes. Appl Environ Microbiol 62(2):725-7.

Ottaviani D, Leoni F, Rocchegiani E, Santarelli S, Canonico C, Masini L, Ditrani V, Carraturo A. 2008. First clinical report of pandemic *Vibrio parahaemolyticus* O3:K6 infection in Italy. J Clin Microbiol 46(6):2144–5.

Pagán R, Mackey B. 2000. Relationship between membrane damage and cell death in pressure-treated *Escherichia coli* cells: differences between exponential- and stationary-phase cells and variation among strains. Appl Environ Microbiol 66:2829-34.

Palou E, Hernández-Salgado C, López-Malo A, Barbosa-Cánovas G.V., Swanson BG, Welti-Chanes J. 2000. High pressure-processed guacamole. Innov Food Sci Emerg Technol 1:69-75.

Pan T, Wang T, Lee C, Chien S, Horng C. 1997. Food-borne disease outbreaks due to bacteria in Taiwan,1986 to 1995. J Clin Microbiol 35(5):1260-2.

Patterson MF. 2005. Microbiology of pressure-treated foods. J Appl Microbiol 98:1400-9.

Patterson MF, Simpson QR, Gilmore A. 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. J Food Prot 58:524-9.

Peffers ASR, Bailey J, Barrow GI, Hobbs BC. 1973. *Vibrio parahaemolyticus* gastroenteritis and international air travel. Lancet 301(7795):143-5.

Phuvasate S, Chen M, Su Y. 2012. Reductions of *Vibrio parahaemolyticus* in Pacific oysters (*Crassostrea gigas*) by depuration at various temperatures. Food Microbiol 31(1):51-6.

Phuvasate S, Su Y. 2013. Impact of water salinity and types of oysters on depuration for reducing *Vibrio parahaemolyticus* in Pacific oysters (*Crassostrea gigas*). Food Control 32(2):569-73.

Potasman I, Paz A, Odeh M. 2002. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. Clin Infect Dis 35:921-8.

Power UF, Collins JK. 1990. Tissue distribution of a coliphage and *Escherichia coli* in mussels after contamination and depuration. Applied and Environmental Microbiology 56:803-7.

Prapaiwong N, Wallace RK, Arias CR. 2009. Bacterial loads and microbial composition in high pressure treated oysters during storage. Int J Food Microbiol 131:145-50.

Presley BJ, Taylor RJ, Boothe PN. 1990. Trace metals in gulf of Mexico oysters. Sci Total Environ 97-98:551-93.

Quevedo AC, Smith JG, Rodrick GE, Wright AC. 2005. Ice immersion as a postharvest treatment of oysters for the reduction of *Vibrio vulnificus*. J Food Prot 68(6):1192-7.

Raghunath P, Acharya S, Bhanumathi A, Karunasagar I, Karunasagar I. 2008. Detection and molecular characterization of *Vibrio parahaemolyticus* isolated from seafood harvested along the southwest coast of India. Food Microbiol 25:824-30.

Ren T, Su Y-C. 2006. Effects of electrolyzed oxidixing water treatment on reducing *Vibrio parahaemolyticus* and *Vibrio vulnificus* in raw oysters. J Food Prot 69(8):1829-34.

Richards GP. 1987. Shellfish-associated enteric virus illness in the United States, 1934-1984. Estuaries 10(1):84-5.

Rippey SR. 1994. Infectious diseases associated with molluscan shellfish consumption. Clin Microbiol Rev 7:419-25.

Ristori CA, Iaria ST, Gelli DS, Rivera IN. 2007. Pathogenic bacteria associated with oysters (*Crassostrea brasiliana*) and estuarine water along the south coast of Brazil. Int J Environ Health Res 17(4):259-69.

Ritz M, Freulet M, Orange N, Federighi M. 2000. Effects of high hydrostatic pressure on membrane proteins of *Salmonella typhimurium*. Int J Food Microbiol 55:115-9.

Ritz M, Tholozan JL, Federighi M, Pilet MF. 2001. Morphological and physiological characterization of *Listeria Monocytogenes* subjected to high hydrostatic pressure. Appl Environ Microbiol 67:2240-7.

Ritz M, Tholozan JL, Federighi M, Pilet MF. 2002. Physiological damages of *Listeria monocytogenes* treated by high hydrostatic pressure. Int J Food Microbiol 79:47-53.

Rivalain N, Roquain J, Demazeau G. 2010. Development of high hydrostatic pressure in biosciences: Pressure effect on biological structures and potential applications in biotechnologies. Biotechnol Adv 28:659-72.

Robert-Pillot A, Guénolé A, Lesne J, Delesmont R, Fournier JM, Quilici ML. 2004. Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. Int J Food Microbiol 91:319-25.

Roque A, Lopez-Joven C, Lacuesta B, Elandaloussi L, Wagley S, Furones MD, Ruiz-Zarzuela I, de Blas I, Rangdale R, Gomez-Gil B. 2009. Detection and identification of *tdh*- and *trh*-positive *Vibrio parahaemolyticus* strains from four species of cultured bivalve molluscs on the Spanish Mediterranean Coast. Appl Environ Microbiol 75(23):7574-7.

Rowse AJ, Fleet GH. 1984. Effects of water temperature and salinity on elimination of *Salmonella charity* and *Escherichia coli* from Sydney rock oysters (*Crassostrea commercialis*). Appl Environ Microbiol 48(5):1061-3.

Sakazaki R, Iwanami S, Fukumi H. 1963. Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus* I. Morphological, cultural and biochemical properties and its taxonomical position. Jpn J Med Sci Biol 16:161-88.

Sakazaki R, Tamura K, Kato T, Obara Y, Yamai S, Hobo K. 1968. Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus* III. Enteropathogenicity. Jpn J Med Sci Biol 21:325-31.

Sale AJ, Gould GW, Hamilton WA. 1970. Inactivation of bacterial spores by hydrostatic pressure. J Gen Microbiol 60:323-34.

San Martín MF, Barbosa-Cánovas GV, Swanson BG. 2002. Food processing by high hydrostatic pressure. Crit Rev Food Sci Nutr 42(6):627-45.

Sato M, Kobori H, Shimada S, Osumi M. 1995. Pressure-stress effects on the ultrastructure of cells of the dimorphic yeast *Candida tropicalis*. Federation of European Microbiological Societies Microbiology Letters 131:11-5.

Schmidt K, Gervelmeyer A. 2003. WHO Surveillance programme for control of foodborne infections and intoxications in Europe 8th report (1999-2000). Available at http://www.bfr.bund.de/internet/8threport/8threp_fr.htm Accessed on May 18, 2014.

Shapiro RL, Altekruse S, Hutwagner L, Bishop R, Hammond R, Wilson S, Ray B, Thompson S, Tauxe RV, Griffin PM, the Vibrio Working group. 1998. The role of Gulf coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988-1996. J Infect Dis 178:752-9.

Shimada S, Andou M, Naito N, Yamada N, Osumi M, Hayashi R. 1993. Effects of hydrostatic pressure on the ultrastructure and leakage of internal substances in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 40:123-31.

Shirai H, Ito H, Hirayama T, Nakamoto Y, Nakabayashi N, Kumagai K, Takeda Y, Nishibuchi M. 1990. Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. Infect Immun 58(11):3568-73.

Singh A, Ramaswamy H. 2013. Effect of high pressure processing on color and textural properties of eggs. J Food Res 2:11-24.

Smelt JPPM. 1998. Recent advances in the microbiology of high pressure processing. Trends Food Sci Technol 9(4):152–8.

Son NT, Fleet GH. 1980. Behavior of pathogenic bacteria in the oyster, *Crassostrea commercialis*, during depuration, re-laying and storage. Appl Environ Microbiol 40:994-1002.

Styles MF, Hoover DG, Farkas DF. 1991. Response of *Listeria monocytogenes* and *Vibrio parahaemolyticus* to high hydrostatic pressure. J Food Sci 56(5):1404-7.

Su H, Chiu S, Tsai J, Lee C, Pan T. 2005. Bacterial food-borne illness outbreaks in northern Taiwan, 1995-2001. J Infect Chemother 11:146-51.

Su Y, Liu C. 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. Food Microbiol 24:549-58.

Su Y, Yang Q, Häse C. 2010. Refrigerated seawater depuration for reducing *Vibrio parahaemolyticus* contamination in Pacific oyster (*Crassostrea gigas*). J Food Prot 73(6):1111-5.

Sujeewa AKW, Norrakiah AS, Laina M. 2009. Prevalence of toxic genes of *Vibrio parahaemolyticus* in shrimps (*Penaeus monodon*) and culture environment. Int Food Res J 16:89-95.

Sun XD, Holley RA. 2010. High hydrostatic pressure effects on the texture of meat and meat products. J Food Sci 75:R17-R23.

Suthienkul O, Ishibashi M, Iida T, Nettip N, Supavej S, Eampokalap B, Makino M, Honda T. 1995. Urease production correlates with possession of the *trh* gene in *Vibrio parahaemolyticus* strains isolated in Thailand. J Infect Dis 172(5):1405-8.

Tada J, Ohashi T, Nishimura N, Shirasaki Y, Ozaki H, Fukushima S, Takano J, Nishibuchi M, Takeda Y. 1992. Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. Mol Cell Probes 6:477-87.

Takahashi A, Kenjyo N, Imura K, Myonsun Y, Honda T. 2000a. Cl⁻ secretion in colonic epithelial cells induced by the *Vibrio parahaemolyticus* hemolytic toxin related to thermostable direct hemolysin. Infect Immun 68(9):5435-8.

Takahashi A, Sato Y, Shiomi Y, Cantarelli VV, Iida T, Lee M, Honda T. 2000b. Mechanisms of chloride secretion induced by thermostable direct haemolysin of *Vibrio parahaemolyticus* in human colonic tissue and a human intestinal epithelial cell line. J Med Microbiol 49(9):801-10.

Takahashi H, Iwade Y, Konuma H, Hara-Kudo Y. 2005. Development of a quantitative real-time PCR method for estimation of the total number of *Vibrio parahaemolyticus* in contaminated shellfish and seawater. J Food Prot 68:1083-8.

Tamplin ML, Capers GM. 1992. Presistence of *Vibrio vulnificus* in tissues of Gulf coast oysters, *Crassostrea virginica*, exposed to seawater disinfected with UV light. Appl Environ Microbiol 58(5):1506-10.

Taniguchi H, Hirano H, Kubomura S, Higashi K, Mizuguchi Y. 1986. Comparison of the nucleotide sequences of the genes for the thermostable direct hemolysin and the

thermolabile hemolysin from *Vibrio parahaemolyticus*. Microb Pathogenesis 1:425-32.

Taniguchi H, Ohta H, Ogawa M, Mizuguchi Y. 1985. Cloning and expression in *Escherichia coli* of *Vibrio parahaemolyticus* thermostable direct hemolysin and thermolabile hemolysin genes. J Bacteriol 162:510-5.

Tholozan JL, Ritz M, Jugiau F, Federighi M, Tissier JP. 2000. Physiological effects of high hydrostatic pressure treatments on *Listeria monocytogenes* and *Salmonella typhimurium*. J Appl Microbiol 88 202-12.

Thompson CA, Jr., Vanderzant C. 1976. Serological and hemolytic characteristics of *Vibrio parahaemolyticus* from marine sources. J Food Sci 41:204-5.

Timoney JF, Abston A. 1984. Accumulation and elimination of *Escherichia coli* and *Salmonella typhimurium* by hard clams in an in vitro system. Appl Environ Microbiol 47(5):986-8.

Tirado C, Schmidt K. 2000. WHO Surveillance programme for control of foodborne infections and intoxications in Europe 7th report (1993-1998). Available at: http://www.bfr.bund.de/internet/7threport/7threp_fr.htm. Accessed on May 18, 2014.

Tuyet DT, Thiem VD, von Seidlein L, Chowdhury A, Park E, Canh DG, Chien BT, Van Tung T, Naficy A, Rao MR, Ali M, Lee H, Sy TH, Nishibuchi M, Clemens J, Trach DD. 2002. Clinical, epidemiological, and socioeconomic analysis of an outbreak of *Vibrio parahaemolyticus* in Khanh Hoa Province, Vietnam. J Infect Dis 186:1615-20.

Utsalo SJ, Eko FO, Antia-Obong EO. 1992. Features of cholera and *Vibrio parahaemolyticus* diarrhoea endemicity in Calabar, Nigeria. Eur J Epidemiol 8(6):856-60.

Vanlint D, Rutten N, Michiels CW, Aertsen A. 2012. Emergence and stability of high-pressure resistance in different food-borne pathogens. Appl Environ Microbiol 78(9):3234-41.

Vasconcelos GJ, Lee JS. 1972. Microbial flora of Pacific oysters (*Crassostrea gigas*) subjected to ultraviolet-irradiated seawater. Appl Microbiol 23:11-6.

Venkateswaran K, Dohmoto N, Harayama S. 1998. Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. Appl Environ Microbiol 64:681-7.

Vuddhakul V, Chowdhury A, Laohaprertthisan V, Pungrasamee P, Patararungrong N, Thianmontri P, Ishibashi M, Matsumoto C, Nishibuchi M. 2000. Isolation of a pandemic O3:K6 clone of a *Vibrio parahaemolyticus* strain from environmental and clinical sources in Thailand. Appl Environ Microbiol 66(6):2685-9.

Vugia DJ, Tabnak F, Newton AE, Hernandez M, Griffin PM. 2013. Impact of 2003 state regulation on raw oyster–associated *Vibrio vulnificus* illnesses and deaths, California, USA. Emerg Infect Dis 19(8).

Wagley S, Koofhethile K, Wing JB, Rangdale R. 2008. Comparison of *V. parahaemolyticus* isolated from seafoods and cases of gastrointestinal disease in the UK. Int J Environ Health Res 18(4):283-93.

Wang C, Huang H, Hsu C, Shyu Y, Yang BB. 2013. Inactivation and morphological damage of *Vibrio parahaemolyticus* treated with high hydrostatic pressure. Food Control 32:348-53.

Wang S, Duan H, Zhang W, Li J. 2007. Analysis of bacterial foodborne disease outbreaks in China between 1994 and 2005. FEMS Immunol Med Microbiol 51:8-13.

Ward L, Bej AK. 2006. Detection of *Vibrio parahaemolyticus* in shellfish by use of multiplex real-time PCR with TagMan fluorescent probes. Appl Environ Microbiol 72:2031-42.

Washington State Department of Health. 2012. Bacteria close some shellfish areas for remainder of summer. New Release. Available at: http://www.doh.wa.gov/Newsroom/2012NewsReleases/12121VibrioUpdate.aspx Accessed on May 17, 2014.

Wong H, Chen M, Liu S, Liu D. 1999. Incidence of highly genetically diversified *Vibrio parahaemolyticus* in seafood imported from Asian countries. Int J Food Microbiol 52:181-8.

Wong H, Liu S, Wang T, Lee C, Chiou C, Liu D, Nishibuchi M, Lee B. 2000. Characteristics of *Vibrio parahaemolyticus* O3:K6 from Asia. Appl Environ Microbiol 66(9):3981-6.

World Health Organization. 1999. *Vibrio parahaemolyticus*, Japan, 1996-1998. Weekly Epidemiological Record 74(43):361-3.

Wouters PC, Glaasker E, Smelt JPPM. 1998. Effects of high pressure on inactivation kinetics and events related to proton efflux in *Lactobacillus plantarum*. Appl Environ Microbiol 64:509-14.

Wuytack EY, Diels AMJ, Michiels CW. 2002. Bacterial inactivation by high-pressure homogenisation and high hydrostatic pressure. Int J Food Microbiol 77:205-12.

Ye M, Huang Y, Chen H. 2012. Inactivation of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oysters by high-hydrostatic pressure and mild heat. Food Microbiol 32(1):179-84.

Ye M, Huang Y, Gurtler JB, Niemira BA, Sites JE, Chen H. 2013. Effects of pre- or post-processing storage conditions on high-hydrostatic pressure inactivation of *Vibrio parahaemolyticus* and *V. vulnificus* in oysters. Int J Food Microbiol 163:146-52.

Ye M, Huang Y, Neetoo H, Shearer AEH, Chen H. 2011. Influence of growth conditions on pressure resistance of *Vibrio parahaemolyticus* in oysters and optimization of post pressure treatment recovery conditions. J Food Prot 74(5):751-8.

Zhang X, Austin B. 2005. Haemolysins in *Vibrio* species. J Appl Microbiol 98:1011-9.

Zimmerman AM, DePaola A, Bowers JC, Krantz JA, Nordstrom JL, Johnson CN, Grimes DJ. 2007. Variability of total and pathogenic *Vibrio parahaemolyticus* densities in northern Gulf of Maxico water and oysters. Appl Environ Microbiol 73:7589-96.