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Title: Refrigerated Seawater Depuration for Reducing *Vibrio parahaemolyticus* Contamination in Raw Pacific Oysters (*Crassostrea gigas*).

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Yi-Cheng Su

Vibrio parahaemolyticus is a seafood-borne pathogen that can cause gastroenteritis in humans. This study investigated the effectiveness of refrigerated seawater (5°C) depuration on reducing *V. parahaemolyticus* in raw Pacific oysters (*Crassostrea gigas*). Raw Pacific oysters were inoculated with a mixed culture of five clinical strains of *V. parahaemolyticus* and depurated with cold seawater (5°C) in a pilot scale recirculating system. The refrigerated seawater depuration was more efficient in reducing *V. parahaemolyticus* contamination in oysters harvested in winter than in summer. Populations of *V. parahaemolyticus* in oysters harvested in winter were reduced by >1.2 log MPN/g after 24 h of depuration in refrigerated seawater. Reductions of *V. parahaemolyticus* in the oysters increased to about 2.3 log MPN/g after 48 h and reached 3.1 log after 96 h of the process. However, it required 144 h of depuration in the refrigerated seawater to achieve a 3-log (MPN/g) reduction of *V. parahaemolyticus* in oysters harvested in summer. The efficacies of refrigerated seawater depuration in reducing *V. parahaemolyticus* were determined at a rate of 0.0211-log/h in oysters harvested in the summer and 0.0362-log/h in oysters harvested

in the winter. This is probably due to the differences between water temperatures of the oyster harvest site (7-9ºC in winter, 16-17ºC in summer) and the refrigerated seawater (5ºC). Because of the increased temperature difference in the summer, it would require a longer time for oysters to adjust their biological activity to the new environment.

Depuration of raw oysters in recirculated refrigerated seawater (5ºC) for up to 144 h did not cause a noticeable fatality of oysters, but increased their ability to survive in subsequent cold storage. The process also reduced fecal coliform contamination in oysters from 10^3 MPN/g to less than 20 MPN/g. When oysters were stored in a refrigerator, 90% of oysters with or without depuration treatment survived after 7 days. However, the survival rate of oysters that had not been depurated in refrigerated seawater dropped sharply from 90 % to 44% after 9 days while 87% of depurated oysters remained alive after the same period of storage. No viable but nonculturable (VBNC) cells of *V. parahaemolyticus* were detected in the oysters depurated in refrigerated seawater for 144 h by reverse transcriptase polymerase chain reaction (RT-PCR) and multiplex PCR.

Refrigerated seawater (5ºC) depuration can be used as a simple and economical post-harvest treatment for reducing *V. parahaemolyticus* contamination in oysters. This process can easily be adopted by the shellfish industry for producing safe oysters for consumers and to reduce *V. parahaemolyticus* infection associated with raw oyster consumption.

Refrigerated Seawater Depuration for Reducing *Vibrio parahaemolyticus*
Contamination in Raw Pacific Oysters (*Crassostrea gigas*)

by
Qianru Yang

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APPROVED:

_____

Major Professor, representing Food Science and Technology

_____

Head of the Department of Food Science and Technology

_____

Dean of the Graduate School

_____

Qianru Yang, Author

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(*Crassostrea gigas*)

Chapter 1

Introduction

Foodborne illness is a common concern among food industries and consumers. The Centers for Disease Control and Prevention (CDC) estimated that 76 million foodborne illnesses occurred in the United States with 325,000 hospitalizations and 5,200 deaths. (Mead and others 1999). Yearly costs of all foodborne diseases in the United States were estimated to be 5-6 billion in direct medical expenses and loss of productivity (NIAID 2000).

Vibrio parahaemolyticus is a foodborne pathogen that naturally inhabits coastal waters and is the main causative agent of human gastrointestinal illness associated with raw seafood consumption. It is estimated that 4500 cases of *V. parahaemolyticus* infection occur each year in the United States (CDC 2008). The first outbreak of *V. parahaemolyticus* in the United States was recorded in 1971 in Maryland. This outbreak involved 425 cases of gastroenteritis associated with consumption of improperly cooked crabs reported in three incidents (Molenda et al. 1972). From 1997 to 1998, more than 700 cases of illness associated with eating raw oysters contaminated with *V. parahaemolyticus* were reported in California, Oregon,

Washington, Connecticut, New Jersey, New York, and British Columbia of Canada (CDC 1998, 1999). In 2004, *V. parahaemolyticus* was the most frequently (51%) reported *Vibrio* species isolated from 240 patients suffering from *Vibrio* infection. Among the patients infected with *V. parahaemolyticus*, 20% were hospitalized and 1% died (CDC 2004).

In 2005, 18% of wound-associated illnesses that occurred in several states after hurricane Katrina were caused by *V. parahaemolyticus* (CDC 2005). Between May 20 and July 31, 2006, the health departments of New York City and the states of New York, Oregon, and Washington reported 177 cases of *Vibrio parahaemolyticus* infection, of which 122 in 17 clusters were associated with restaurants, seafood markets, or recreational harvesting. The numbers of confirmed cases (72) in this report were more than the average number (9) reported during summer months(May, June, and July) from 2000 to 2004 in the entire United States (CDC 2006). The unexpected outbreak renewed the importance of *V. parahaemolyticus* contamination in oysters a major public health concern. Beginning in 2007, Centers for Disease Control and Prevention (CDC) required state health departments to report illness caused by *V. parahaemolyticus* and other *Vibrio* species, and the data are now summarized annually (CDC 2008). The development of effective post-harvest treatments to eliminate *V. parahaemolyticus* contamination is an important step to reduce risk of *V. parahaemolyticus* infection associated with seafood consumption, particularly in the case of raw oysters.

Several processes, including freezing (Jonhson and Brown 2002), low temperature pasteurization (Andrews and others), high pressure processing (He and others 2002), and irradiation (Andrews and others 2003), have been reported to be capable of reducing *V. parahaemolyticus* in oysters. However, most of these treatments require specific equipment or facilities and the oysters are often killed during processing. Cost-effective post-harvest processing for reducing *V. parahaemolyticus* in raw oysters without adverse effects on oysters remains to be developed.

Depuration is a process that allows shellfish to purge sand and grit from the gut into clean seawater. (Blogoslawski and Stewart 1983). It has a long history (more than 75 years) as a post-harvest treatment to reduce microbial contaminants in shellfish (Canzonier 1991). Unfortunately, studies have reported that the process, normally carried out at ambient temperatures, was not effective in reducing *Vibrio* contamination in oysters (Colwell and Liston 1960, Vasconcelos and Lee 1972, Eyles and Davey 1984). However, lowering the depuration temperature might increase the efficacy of the process for decontaminating *V. parahaemolyticus* in shellfish. Several studies of occurrence of *V. parahaemolyticus* in the marine environments have demonstrated that densities of *V. parahaemolyticus* in seawater were positively correlated with water temperatures (Kaneko and Colwell 1973, DePaola and others 1990, Duan and Su 2005). The present study was conducted to determine the potential use of refrigerated seawater depuration for decontaminating *V. parahaemolyticus* in raw oysters.

During the refrigerated seawater depuration process, *V. parahaemolyticus* in oysters encounters an unfavorable growth environment and might enter the viable but nonculturable (VBNC) state as a survival strategy (Jiang and Chai 1996). Once it is exposed to favorable growth conditions, such as >20ºC, VBNC *V. parahaemolyticus* can recover and multiply sufficient levels to cause disease (Coutard and others 2007). To investigate the possibility of *V. parahaemolyticus* entering the VBNC state during refrigerated seawater depuration, a reverse-transcriptase polymerase chain reaction (RT-PCR) (Coutard and others 2005) was used to analyze oyster samples after the process. In addition, shelf life of oysters stored at refrigeration temperatures after the depuration was studied.

Chapter 2

Literature Review

Vibrio parahaemolyticus is a gram-negative bacterium that occurs naturally in the marine environment. This human pathogen is frequently found in shellfish and can cause acute gastroenteritis characterized by diarrhea, vomiting, and abdominal cramps through consumption of contaminated raw fish or shellfish (Rippey 1994). This organism was first identified as a causative agent of food-borne gastroenteritis after a large outbreak (272 illnesses and 20 deaths) associated with consumption of sardines was reported in Japan in 1951 (Fujino and others 1953). Since then, *V. parahaemolyticus* has been recognized a leading cause of illness associated with seafood consumption (Daniel et al. 2000, Honda and Iida 1993) and has been isolated from marine environmental samples as well as a variety of seafood in many countries around the world, including China (Aoki and others 1967), Korea (Chun and others 1967), Thailand (Pan-Uraiand others 1973), Indonesia (Joseph 1974), Vietnam (Neumann and others 1972.), India (Chatterjee and Sen 1974, Saldanha and others 1975, Nair and others 1980), Russia (Libinzonand others 1977), Australia (Wallace and Battey 1971, Sutton 1974), Great Britain (Ayres and Barrow 1978, Barrow and Miller 1972, Hechelmann and others 1971), France (Robert-Pillot and others 2004), Germany (Leistner and Hechelmann 1974), Italy (Cabassi and Mori 1976, Realiand others 1977), Canada (Thompson and Trenholm 1971, Thomson and Thacker 1972, Varga and Hirtle 1972) and the United States (CDC 1998, 1999).

The first outbreak of *V. parahaemolyticus* in the United States was reported in 1971. Three incidents involving 425 cases of gastroenteritis associated with consumption of improperly cooked crabs occurred in Maryland (Molenda et al. 1972).

Now, outbreaks of *V. parahaemolyticus* infections are frequently reported around the world, including in North America (CDC 1998, 1999, 2006; Gil et al. 2007), Asia (Lee 2007), and Europe (Vernocchi 2007, Pinto 2007). Therefore, illness caused by *V. parahaemolyticus* infection is a global health concern. Reducing *V. parahaemolyticus* contamination in seafood post-harvest is an important step towards the risk of *V. parahaemolyticus* infection associated with seafood consumption.

2.1 Ecology of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is widely distributed in the coastal waters around the world. It can be found in the water, sediment, suspended particles, plankton (Thompson 1976, Joseph and others 1982), and 30 different marine species, including eel, crab, clams, oysters, lobsters, scallops, sardines, shrimp, and squid (Fishbein and others 1974). This bacterium can grow in environments containing salt (NaCl) concentrations ranging from 1 to 8% with an optimal growth condition of 2-4% of salt (Sakazaki 1979). The optimal growth temperature for *V. parahaemolyticus* is between 30 and 35°C with an upper growth limit of 45.3°C (Sakazaki 1983). *V. parahaemolyticus* can grow over a wide pH range between 4.8 to 11.0 with an optimal range between 7.6 and 8.6. (Beuchat, 1973, Sakazaki 1983) In some extreme conditions, such as starvation, *V. parahaemolyticus* can enter a viable but non-culturable (VBNC) state (Jiang and Chai 1996).

Vibrio parahaemolyticus is sensitive to low temperatures and is unable to grow at hydrostatic pressures of 200 atm or higher, as encountered in the deep sea (Schwartz

and Colwell 1974). Therefore, it is more commonly isolated from shallow water and estuarine environments. Several studies have reported that the distribution of *V. parahaemolyticus* in the marine environments is affected by the water temperatures. In an early study conducted by Kaneko and Colwell (1973), *V. parahaemolyticus* was not detected in the waters of Chesapeake Bay, Maryland during winter time, but survived in sediment. When the water temperature rose to 15ºC in summer, *V. parahaemolyticus* was released from the sediment into water. The study reported an increase of the densities of *V. parahaemolyticus* in water up to 1,000 cells/100 ml when water temperatures rose to 25 to 30°C in August. A recent study investigating occurrence of *V. parahaemolyticus* in Yaquina and Tillamook Bays in Oregon between 2002 and 2003 also reported that densities of *V. parahaemolyticus* in seawater and sediment were positively correlated to water temperature with higher densities detected in summer, especially July and August (Duan and Su 2005).

The increase in *V. parahaemolyticus* density in water when temperature arises also means a greater possibility of fish and shellfish being contaminated with this bacterium. Therefore, it is more likely to detect *V. parahaemolyticus* in fish and shellfish harvested in spring and summer than in winter. An early study conducted at the coast of Sydney reported that the viable counts of *V. parahaemolyticus* in oyster increased with increasing water temperature, from non-detectable level (< 3.0 MPN/100g) at 10ºC to 1.5 log MPN/g at 22ºC (Sutton 1974). Another study analyzing populations of *V. parahaemolyticus* in oysters harvested at the Galveston Bay reported relatively high levels of *V. parahaemolyticus* in oysters (100 to 1,000 per gram) when

water temperature was between 27.8 to 31.7°C during the summer (DePaola and others 2000). In addition, higher levels of *V. parahaemolyticus* (20-43 MPN/g) were reported in oysters harvested in July and August at the Tillamook and Yaquina Bays in Oregon when compared with levels in oysters (<3 MPN/g) harvested in winter (Duan and Su 2005).

Recently, virulent strains of *V. parahaemolyticus* were isolated from 48 of 72 raw oysters collected from Korean retail outlets between April and December with the highest level of *V. parahaemolyticus* in oysters detected in August and September (Lee and others 2007). These results indicate that consumption of raw fish or shellfish, particularly oyster, harvested from water with a temperature higher than 14ºC could result in development of gastroenteritis caused by *V. parahaemolyticus* infection. This was demonstrated by a surprise outbreak of *V. parahaemolyticus* gastroenteritis associated with eating raw Alaskan oysters in the summer of 2004. A total of 14 on board a cruise ship in Alaska got sick after eating raw oyster contaminated with *V. parahaemolyticus* serotype O6:K18. Prior to the outbreak, the water in Alaskan Gulf was considered too cold to support *V. parahaemolyticus* to grow to a level that is high enough to produce detectable contamination in Alaskan oysters (Joseph and others 2005). In the history of Alaska, a nonpathogenic strain of *V. parahaemolyticus* was isolated from the environment only once, in 1974 (Vasconcelos and others 1975). From 1995 to 2003, no *V. parahaemolyticus* was detected from approximate 400 Alaska oyster or marine environmental samples. However, a gradual increase in water temperature was recorded in some Alaskan oyster farms in recent years. The mean

water temperature at the oyster farm whose oysters were implicated in the 2004 outbreak had increased 0.21ºC every summer since 1997. During the summer of 2004, the water temperature rose to higher than 15ºC and remained through July and August, which led to the first ever and unexpected outbreak of *V. parahaemolyticus* infection in Alaska (McLaughlin and others 2005).

2.2 Epidemiology of *Vibrio parahaemolyticus*

Seafood is the main vehicle for foodborne illness caused by *Vibrio parahaemolyticus* infection. Although fish has been reported as the primary vehicle for infection in Japan, outbreaks in the United States have been more frequently associated with raw shellfish consumption (Barker and others 1975, Fujino and others 1972). Shellfish are filter-feeding bivalves. They filter water for nutrients and, at the same time, accumulated microorganisms, including *Vibrio* spp., in the digestive tract.

While all shellfish, including oyster, lobster, mussel, clam, shrimp, and crab, could be contaminated with *Vibrio* spp. at harvest, the risk of *Vibrio* infection is usually in association with oyster consumption because oyster is the most abundant shellfish harvested around the world and is commonly consumed raw (FAO 1998). In the United States, more than 27 million pounds of oysters are harvested each year and most of them are sold live or shucked without further processing (Hardesty 2001). Consumption of raw oysters was reported responsible for about 95% of all deaths associated with seafood consumption in the United States (Oliver 1989). It was estimated that one in 2,000 meals of raw molluscan shellfish serves as the vehicle for

Vibrio infection (Ahmed 1991). In the U.S, more than 700 cases of illness caused by *V. parahaemolyticus* associated with raw oyster consumption were reported between 1997 and 1998 (DePaola and others 2000). Between May 20 and July 31, 2006, the health departments of New York City and the states of New York, Oregon, and Washington reported 177 cases of *Vibrio parahaemolyticus* infection (CDC 2006), of which 122 were associated in 17 clusters with restaurants, seafood markets, or recreational harvesting.

2.3 Virulence factors of *Vibrio parahaemolyticus*

2.3.1 Hemolysins

Although *V. parahaemolyticus* is a major cause of gastroenteritis associated with raw seafood consumption, most existing strains of *V. parahaemolyticus* in nature are nonpathogenic to humans (Nishibuchi and Kaper 1995). A Japanese researcher, Wagatsuma, invented a special blood agar medium for testing the hemolytic characteristics of *V. parahaemolyticus* in 1968. This agar is capable of identifying strains of *V. parahaemolyticus* based on hemolytic activity with the hemolytic strains of *V. parahaemolyticus* being identified through formation of a clear halo surrounding colonies after 18 to 24 h of incubation of the agar plates at 37ºC (Joseph and others 1982, Miyamoto and others 1969). The ability of *V. parahaemolyticus* to produce a beta-type hemolysis on the Wagatsuma blood agar was named Kanagawa phenomenon (KP) (Sakazaki and others 1968). Early epidemiological studies found that strains of *V. parahaemolyticus* isolated from people suffering gastroenteritis were

usually hemolytic, while most strains isolated from environmental samples were not (Yeung and Boor 2004). Clinical studies have confirmed that almost all the strains of *V. parahaemolyticus* isolated from patients with gastroenteritis are KP-positive, whereas environmental isolates are rarely KP-positive. The hemolysin responsible for KP was later identified and named thermostable direct hemolysin (TDH) because it remained active after a heat treatment at 100ºC for 15 min (Fukui and others 2005). TDH was the first recognized virulence factor for *V. parahaemolyticus* and has been used as an important marker for identifying virulent strains (Cook and others 2002; Okuda and others 1997).

Purified TDH is a protein that consists of 165 amino acids and has a molecular weight of ca. 44,000 daltons (Miyamoto and others 1980). It has been reported to be cytotoxic, enterotoxic and cardiotoxic in experimental animals (Honda and Iida 1993, Jay and others 2005). It damages the erythrocyte membrane by acting as a pore-forming toxin that alters ion flux in intestinal cells and leads to a secretory response and diarrhea (Zhang and Austin 2005). The gene encoding TDH (*tdh*) was first cloned by Kaper and others (1984) and sequenced in 1985 (Nishibuchi and Kaper 1985).

While epidemiological investigation has revealed a strong tie between the Kanagawa phenomenon and *V. parahaemolyticus* pathogenicity, KP-negative strains have also been isolated from outbreak patients. Honda and others (1988) reported isolation of a KP-negative *V. parahaemolyticus* strain from an outbreak of gastroenteritis in the Republic of Maldives in 1985. The strain did not carry the *tdh* gene, but produced a TDH-related hemolysin (TRH). Analysis of amino acid

sequences of TRH revealed about 67% homology to TDH, and TRH was found to be biologically similar but physiochemically different compared to TDH. Unlike the thermostable TDH, TRH is labile to a heat treatment at 60ºC for 10 min (Honda and Iida 1993). The gene (*trh*) encoding TRH has also been cloned and sequenced (Kishishita and others 1992). Two subgroups, *trh*1 and *trh*2, which shared 84% sequence identity and were 54.8 to 68.8% homologous to the *tdh* gene were reported. Similar to TDH, TRH can induce chloride secretion in human colonic epithelial cells and is, therefore, considered a virulence factor of *V. parahaemolyticus* (Takahashi and others 2000a, b). A survey of 285 strains of *V. parahaemolyticus* revealed that the *trh*-positive strains had a strong association with gastroenteritis (Shirai and others 1990). Today, both TDH and TRH are recognized virulence factors of *V. parahaemolyticus* capable of causing gastroenteritis in human.

2.3.2 Urease production

In general, strains of *V. parahaemolyticus* do not produce urease (Osawa and other 1996, Okuda and others 1997). However, some urease-producing strains have been reported by several investigators (Chitu and others 1977, Joseph and others 1982). Kelly and Stroh (1989) reported that clinical isolates from patients with gastroenteritis in Canada were all Uh[+] strains, but none of them were positive for Kanagawa phenomenon. Clinical strains isolated from the 1997 outbreaks that occurred in the Pacific Northwest region of the United State were also identified to be urease positive and possessed both the *tdh* and *trh* genes (CDC 1998). Urease activity was proposed to

be a simple screening test for pathogenic strains (Kaysner and others 1994). However, other studies reported that the urease activity can not be used for predicting virulent strains.

In an investigation of 132 strains of *V. parahaemolyticus* isolated from patients and suspected food items of foodborne cases in Kanagawa Prefecture area in Japan, only 10 strains could produce urease and 4 of them did not produce TDH (*tdh*⁻). A total of 106 strains were identified *tdh*⁺ with only 6 strains (<6%) were urease positive (Uh⁺) while all 5 *trh*⁺ strains were reported Uh⁺. Another study analyzing 60 Uh⁺ strains of *V. parahaemolyticus* isolated from outbreaks occurred on the West Coast of the U. S. from 1979 to 1995 revealed that 98% of them carried either *trh1* or *trh2* while 90% of them carried *tdh* (Okuda and others 1997). On the other hand, 80% of 25 Uh⁻ strains had the *tdh* gene, but none of them had the *trh* gene. Taken together, these results indicate that urea hydrolysis might be a reliable indicator for identifying *trh*⁺ strains but not for *tdh*⁺ strains (Osawa and others 1996). Recently, the relationship between urease and TRH activity has been reported to be due to a genetic linkage between the urease gene (*ureC*) and *trh* on a chromosome of virulent *V. parahaemolyticus* strains (Park and others 2000; Iida and others 1997). However, the urease gene cluster has no influence on the regulation of *tdh* or *trh* gene expression (Nakaguchi 2003).

2.3.3 Other virulence factors

One important virulence factor of Gram-negative bacteria is the adherence to epithelial cells of the hosts. It has been reported that *V. parahaemolyticus* may produce cell-associated hemagglutinins when adhered to intestinal mucosa (Yamamoto and Yokota 1989). Hemagglutinin (HA) is an antigenic glycoprotein which is responsible for binding virus to the cell that is being infected. The protein can also cause red blood cells to clump together. In addition, the pili of *V. parahaemolyticus* might also play a role in adhering to the epithelial receptor of the intestine (Nakasone and Iwanaga 1990). It is speculated that the adhesive processes may well be involved in diseases caused by *V. parahaemolyticus*. However, no evidence has been found to demonstrate that adherence to human epithelium is a virulence factor of pathogenic *V. parahaemolyticus* (Reyes and others 1983).

V. parahaemolyticus requires iron as an essential element for growth (Guerinot 1994). It can produce a novel siderophore named vibrioferrin under conditions of little or no iron, which facilitates iron acquisition and utilizes heme to create a new iron source for the growth (Yamamoto and others 1994, 1995). Yamamoto and others (1999) reported that higher levels of vibrioferrin in the nutrient-depleted culture supernatant were produced by clinical isolates of *V. parahaemolyticus* than by isolates from food or environmental sources when they were grown in a medium containing limited iron. Other studies reported that *V. parahaemolyticus* cultures demonstrated greater adherence, increased hemolytic activities, enhanced lethality for mice, and higher proliferation rates when grown in iron-limited media (Dai and others 1992,

Wong and Lee 1994). These studies indicate that synthesis of vibrioferrin under iron-limited conditions might contribute to the pathogenesis of *V. parahaemolyticus*.

Although TDH and TRH have been recognized as the major virulence factors of *V. parahaemolyticus*, strains of *V. parahaemolyticus* that do not produce TDH or TRH have recently been reported to be capable of inducing fluid accumulation in suckling mice (Kothary and others 2000). However, there is no report of isolating this type of V. *parahaemolyticus* from clinical and environmental samples yet. Virulence factors of *V. parahaemolyticus* other than TDH and TRH may exist and need to be identified. For example, a heat-labile protein (serine protease) produced by a clinical *V. parahaemolyticus* strain carrying neither *tdh* nor *trh* gene was suspected as a potential virulence factor (Lee and others 2002). The purified protease had significant inhibitory effects on the growth of Chinese hamster ovary, HeLa, Vero, and Caco-2 cells. It could cause erythrocyte lysis, tissue hemorrhaging, and lead to death when injected into mice either intraperitoneally or intravenously.

2.4 Symptoms of *Vibrio parahaemolyticus* infection

The most common symptoms of *V. parahaemolyticus* infection are abdominal pain and diarrhea. In severe cases, watery diarrhea containing mucus and blood may occur. Persons with *V. parahaemolyticus* infection may also have symptoms of low-grade fever, vomiting, nausea, general fatigue, headache and chill. Dehydration, collapse, and abnormality on electrocardiograms have occurred in individual cases (Joseph and others 1982, Honda and Iida 1993, Carpenter 1995). A summary of

common clinical symptoms associated with *V. parahaemolyticus* gastroenteritis infection is presented in Table 2.1 (FDA 2005a). The incubation time of the infection usually ranges from 4 to 96 h (Twedt 1989) and the clinical symptoms of the infection may last for 2 to 6 days. While *V. parahaemolyticus* infection is usually self-limiting, in severe cases, it may lead to development of septicemia and become life-threatening to people having underlying medical conditions such as liver disease or immune disorders. In addition, *V. parahaemolyticus* also can cause wound infection in people exposed to contaminated seawater (Bonner and others 1983, Murray and others 1998). Two deaths were reported among three cases of wound infections caused by *V. parahaemolyticus* in Louisiana and Mississippi after Hurricane Katrina in 2005 (CDC 2005).

Table 2.1. Common clinical symptoms associated with gastroenteritis caused by *Vibrio* spp. (Data adapted from Barker and Gangarosa 1974; Levine and others 1993)

Symptoms	Incidence of Symptoms	
	Median	**Range**
Diarrhea	98%	80 to 100%
Abdominal cramps	82%	68 to 100%
Nausea	71%	40 to 100%
Vomiting	52%	17 to 79%
Headache	42%	13 to 56%
Fever	27%	21 to 33%
Chills	24%	4 to 56%

2.5 Incidence of *Vibrio parahaemolyticus* infection

The history of *Vibrio parahaemolyticus* can be traced back to the middle of the twentieth century (Fujino and others 1953). Since then, *V. parahaemolyticus* has been a causative agent responsible for 20-30% of all food poisoning cases in Japan (Alam and others 2002) and a well-documented common cause of seafood-borne illness throughout Asia (Chen and others 1991; Yamamoto and other 1992; Deepanjali and others 2005). *V. parahaemolyticus* accounted for 69% of total bacterial foodborne outbreaks (1,495 cases) in Taiwan from 1981 to 2003 (DOH 2005). In Japan, over 70% of the seafood poisoning cases were related to *V. parahaemolyticus* (Sakazaki 1967) and *V. parahaemolyticus* was the leading cause of 1,710 food poisoning incidents (24,373 cases) reported in Japan between 1996 and 1998 (IDSC 1999). Although raw or partially cooked seafood is usually the main vehicle for *V. parahaemolyticus* infection (Martinez-Urtaza and others 2004), illnesses associated with cooked products contaminated with *V. parahaemolyticus* have also been reported. Cooked foods contaminated with *V. parahaemolyticus* were responsible for 31.1% of 5,770 foodborne outbreaks occurred in China from 1991 to 2001 (Liu and others 2004).

Compared to Asian countries, infections of *V. parahaemolyticus* are less frequently reported in European countries, though sporadic outbreaks have been reported in some countries. Eight cases of *V. parahaemolyticus* gastroenteritis related to fish or shellfish ingestion occurred in Spain 1989 (Martinez-Urtaza and others 2004). A serious outbreak affecting 44 patients associated with consumption of Asian

imported shrimps was reported in France in 1997 (Robert-Pillot and others 2004). One outbreak involving 64 cases of illness associated with raw oyster consumption was reported in Galicia, Spain, in 1999 (Lozano-León and others 2003). Another *V. parahaemolyticus* outbreak of 80 illnesses also occurred in A Coruña, Spain in July 2004 after wedding guests ate at a restaurant. Epidemiologic investigation of the outbreak identified the boiled crab consumed at the wedding as the most possible vehicle of infection. The crabs were processed under unsanitary conditions and held at room temperature for several hours before they were served (Martinez-Urtaza and others 2005).

In the United States, *V. parahaemolyticus* was first identified as an etiological agent of food-related gastroenteritis after three foodborne outbreaks involving 425 illnesses associated with consumption of improperly cooked crabs occurred in Maryland in August 1971 (Molenda and others 1972). Since then, approximately 40 outbreaks of *V. parahaemolyticus* infections were reported to the Centers for Disease Control and Prevention (CDC) between 1973 and 1998, with most of the illnesses related to shellfish consumption (Daniels and others 2000). Among them, the largest outbreak of *V. parahaemolyticus* gastroenteritis in United States history was reported in the summer of 1978, when 1,133 of 1,700 persons attending a dinner in Port Allen, LA were infected (Montville and Matthews 2005). In addition, four major outbreaks of *V. parahaemolyticus* infections resulting in more than 700 cases of illness occurred in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions of the United States in 1997 and 1998. The first one occurred in summer 1997 in the Pacific

Northwest (Oregon, Washington, California and British Columbia of Canada) with a total of 209 cases and one death (CDC 1998). Nearly all the cases were associated with eating raw oysters harvested in the State of Washington and British Columbia, Canada. In 1998, two outbreaks involving 43 and 416 cases in the states of Washington and Texas, respectively, were also linked to raw oyster consumption (DePaola and others 2000). Following the Texas outbreak, the fourth outbreak of eight cases of *V. parahaemolyticus* infection associated with eating raw oysters and clams harvested from Oyster Bay off New York's Long Island Sound was reported in Connecticut, New Jersey, and New York between July and September 1998 (CDC 1999). More recently, an outbreak of *V. parahaemolyticus* infection caused by Alaska oysters occurred on board a cruise ship in Alaska in the summer of 2004 (McLaughlin and others 2005). Recently, a *V. parahaemolyticus* outbreak of 177 cases (72 confirmed and 105 probable) occurred in New York City, New York State, Oregon and Washington in the summer of 2006 and was linked to consumption of contaminated oysters harvested in Washington and British Columbia (CDC 2006). The number of confirmed cases in this outbreak (n=72) was greater than the annual average of 16 confirmed cases reported to CDC in New York, Oregon and Washington and the average number reported in the entire United States from 2000 to 2004. The occurrence of these outbreaks over several geographical locations indicates that contamination of *V. parahaemolyticus* in oysters is a food safety concern in the United States.

2.6 Prevention of *Vibrio parahaemolyticus* infection

2.6.1   Regulation and Education

Following the four outbreaks that occurred in 1997 and 1998, the United States Food and Drug Administration (FDA) initiated a risk assessment on controlling infection of *V. parahaemolyticus* transmitted by raw oysters. In May 1999, FDA announced its intent to conduct a risk assessment of the public health impact of *V. parahaemolyticus* in raw molluscan shellfish in the *Federal Register*. The risk assessment went through the complete judicial process and a draft risk assessment report on the estimated public health risks associated with raw oysters containing pathogenic *Vibrio parahaemolyticus* was announced in 2001 (FDA 2001). The objectives of the risk assessment were: (a) to create a mathematical model and assess the current risk of becoming ill due to consumption of pathogenic *V. parahaemolyticus* in raw oysters, and (b) to develop a comprehensive and current scientific framework, which would assist the agency with the review of current programs relating to the regulation of *V. parahaemolyticus* in raw molluscan shellfish to ensure that such programs protect the public health. The risk assessment task force was also charged to evaluate the evidence for increased risks from specific newly emerging "outbreak strains", the effectiveness of potential strategies for limiting exposure of the public to raw molluscan shellfish (particularly oysters containing pathogenic *V. parahaemolyticus*), the current criteria for opening and closing harvest waters, and FDA's previous established guideline level of 10,000 *V. parahaemolyticus* per gram of food (FDA 1997).

The levels of *V. parahaemolyticus* in oysters at the time of consumption depend on methods of harvesting and post-harvest handling. It has been reported that the intertidal harvest in Pacific Northwest estuaries had significant effects on the levels of *V. parahaemolyticus* in oysters (Nordstrom and others 2004). In the process of intertidal harvest, oysters are first placed into baskets at low tide and then harvested by boats when the tide rises. The oysters are usually exposed to ambient air for several hours until being shipped to plants for processing. Such exposure of oysters to temperatures that are much higher than the water temperature allows *V. parahaemolyticus* to proliferate rapidly in oysters, especially on a warm day. Nordstrom and others (2004) reported that total populations of *V. parahaemolyticus* in oysters increased 4- to 8- fold while the *tdh*-positive *V. parahaemolyticus* counts also increased from ≤10 to as high as 160 CFU/g after being exposed to ambient air between tides. The study also demonstrated that an overnight submersion for a single tidal cycle could reduce *V. parahaemolyticus* levels similar to those determined prior to the intertidal exposure. Therefore, intertidal harvest of oysters should be conducted at high tide to avoid exposure of oysters to air temperatures before harvest.

In addition to harvest methods, post-harvest handling also affects the levels of *V. parahaemolyticus* in oysters. The processes may vary in different geographic areas and at different times of year. For example, holding time for oysters after harvest and before refrigeration (unrefrigerated storage) varies. Although the density of *V. parahaemolyticus* in oysters is usually lower than 10^3 CFU/g at harvest (Kaysner and DePaola 2000), *V. parahaemolyticus* can multiply rapidly in oysters upon exposure to

elevated temperatures. Populations of *V. parahaemolyticus* on the surface of the freshly landed fish could multiply from less than 10^2 CFU/cm^2 to $10^{5\text{-}6}$ CFU/cm^2 after holding the fish at 21-25 ºC for 10 h (Asakawa 1974). Therefore, harvested oysters should be stored at refrigeration temperature as soon as possible to prevent rapid growth of *V. parahaemolyticus* in contaminated oysters. The FDA's risk assessment report (2001) has described how *V. parahaemolyticus* may proliferate to an infective dose upon exposure of contaminated oysters to warm temperatures before consumption.

In 1997, FDA established a guidance limit of 10,000 viable cells per gram for *V. parahaemolyticus* in shellfish. However, epidemiological investigation of the four outbreaks that occurred between 1997 and 1998 indicated that fewer than 10,000 *V. parahaemolyticus* per gram were present in oysters of implicated harvest areas. The overall levels of *V. parahaemolyticus* found in some oysters from implicated harvest sites were lower than 1,000 and some of them were as low as 100 cells per gram (Kaysner and DePaola 2000). Since not all *V. parahaemolyticus* strains are pathogenic to humans, the FDA's risk assessment report suggested tests of pathogenic *V. parahaemolyticus* in oysters at harvest as a further step to protect consumers from *V. parahaemolyticus* illness associated with raw oyster consumption. Recently, the National Shellfish Sanitation Program, Guide for the control of Molluscan Shellfish (FDA 2005b) also established time-to-temperature regulations that limit the time oysters are held at ambient temperatures prior to refrigeration (Table 2.2). The temperature control is defined as the management of shellstock temperatures by means

of ice, mechanical refrigeration or other approved means which is capable of lowering

temperature of the shellstock to 50 °F (10 °C) or lower.

Table 2.2 Time-to-temperature regulations for *Vibrio parahaemolyticus* (Data adapted
from FDA 2005b).

Action Level	Average Monthly Maximum Air Temperature	Maximum Hours from Harvest to Temperature Control
Level 1	<66 °F (18 °C)	36 hours
Level 2	66 °F – 80 °F (19 °C-27°C)	12 hours
Level 3	≥81 °F (≥27 °C)	10 hours

Other than government regulation and guidance, consumer education, such as

consumer bulletins, health workshops and lectures, can also help reduce risk of *V.*

parahaemolyticus infection associated with seafood consumption. Unfortunately,

those actions only led to industrial loss in the Gulf of Mexico because consumers were

scared by the warning and stopped eating raw shellfish, but there was little impact on

the number of illnesses and death (Andrews 2004). However, there are no doubts that

the risk of *V. parahaemolyticus* infection can be reduced through public education on

proper handling of foods, including holding seafood at a sufficiently low temperature

to prevent *V. parahaemolyticus* from rapid multiplication, thorough cooking of raw

seafood to destroy *V. parahaemolyticus* and avoiding recontamination of cooked foods

(Powell 1999). It is estimated that more than 60% of seafood-associated illness could

be avoided if consumers would stop eating raw or undercooked molluscan shellfish

(Liston 1990). People in high risk groups, for example, those who have liver disease,

should be informed of potential risks associated with consumption of raw or undercooked seafood and possible life-threatening disease caused by *Vibrio* infection.

2.6.2 Post-harvest processes

Refrigeration has been the most commonly used method for preserving quality and extending shelf life of shellstock and shucked oysters (Andrews 2004). However, other methods, such as use of chemicals, heating, high pressure processing and irradiation may also be used.

2.6.2.1 Natural Chemicals

The optimal pH range for growth of *V. parahaemolyticus* is between 7.6 and 8.6 (Sakazaki 1983). Acetic acid and citric acid are commonly consumed with raw oysters when hot sauces and lemon juice are used for flavoring. These natural chemicals have been reported to exhibit certain degrees of inhibitory effects on growth of bacteria. It has been reported that the number of *V. vulnificus* cells on oyster meat surface could be reduced from 1.3×10^4 CFU/oyster to undetectable levels (< 10 CFU/oyster) after covering the half-shell oysters with hot sauce for 10 min (Sun and Oliver 1995). Castillo and others (2000) reported that *V. cholerae* (10^8 CFU/ml) in broth culture was completely inactivated in freshly squeezed lemon juice (1:100 dilution) within 5 min. Similarly, populations of *V. vulnificus* in broth culture could be reduced from 4.4 log CFU/mL to 2.5 and 1.3 log CFU/mL after being treated with 50 and 100% lemon juice for 30 min, respectively (Andrews 2004).

2.6.2.2 Thermal Processes

Thermal processes including heating (low temperature pasteurization) and cooling (cold storage), icing and freezing have been reported to be capable of achieving certain reductions of *Vibrio* species in oysters.

V. parahaemolyticus is sensitive to heat. Johnson and Brown (2002) reported that heating a culture suspension at 70°C for 2 min resulted in greater than 7-log reductions of *V. parahaemolyticus*. A low-temperature pasteurization of banded in-shell oysters at 52°C water for 10 min to achieve an internal temperature of 48-50°C for 5 min was reported capable of reducing numbers of *V. vulnificus* and *V. parahaemolyticus* in oysters by 99.9% and to non-detectable levels, respectively (Andrews and others 2000). However, some strains of *V. parahaemolyticus* required nearly 9 min of holding inoculated oysters at 55°C to achieve a 5-log reduction in populations (Johnson and Brown, 2002). The pasteurized oysters had a raw-like quality as long as the pasteurization temperature did not exceed 52.5°C and could be stored in ice for up to three weeks. However, the pasteurization process may change the texture of oyster meat due to protein denaturation caused by the heat treatment.

Cold storage including refrigeration, icing and freezing is the most effective means to prevent growth of microorganisms in food. Currently, the National Shellfish Sanitation Program Manual of Operations recommends that harvested clams be cooled down to 7.2°C (45°F) within 20 h of harvest during summer, maintained at that temperature or lower during storage, and shipped at or below temperatures of 10°C (50°F) (NSSPMO 1995).

Thompson and Vanderzant (1976) found that storing shucked oysters at 3°C for 7 days resulted in decreases of *V. parahaemolyticus* in oysters from >11,000 to 0.36 MPN/g. Quevedo and others (2005) reported that populations of *V. vulnificus* in shellstock oysters immersed in ice for 3 h and then stored in a refrigerator gradually decreased from 2.9 CFU/g to 1.6 and 1.2 log CFU/g after 7 and 14 days of storage, respectively. However, total heterotrophic bacteria and fecal coliform in oysters increased from 4.6 CFU/g and 1.3 MPN/100g to 5.5 log CFU/g and 4.8 MPN/100g in oysters, respectively, after 14 days.

Frozen storage is a method commonly used to preserve product quality by inhibiting growth of bacteria and has been reported capable of achieving certain degrees of reductions of *V. parahaemolyticus* in oyster meat and half-shell oysters. Muntada-Garriga and others (1995) reported that *V. parahaemolyticus* in oyster homogenates (10^{5-7} CFU/g) could be completely inactivated after storage at -18 and -24°C for 15 to 28 weeks. Frozen storage of half-shell oysters for up to 4 months at -20°C was reported capable of reducing low populations of *V. parahaemolyticus* (<1,000 CFU/g) to non-detectable levels (Andrews 2004). However, the process could not reduce high levels of *V. parahaemolyticus* (>10^3 CFU/g) in oysters to non-detectable levels within 6 months.

2.6.2.3 High pressure processing

High pressure processing (HPP) is a non-thermal process that can destroy bacterial cells by inactivating enzymes and altering membrane permeability (He and

others 2002). Several studies have shown that HPP could be used to inactivate *V. parahaemolyticus* in oysters. Most environmental strains of *V. parahaemolyticus* were totally inactivated at 300 MPa for 300s (Chen, 2007). However, clinical strains are known to be more resistant than the environmental strains. HPP treatment of 300 MPa for 180 s was required to achieve a >5-log reduction of clinical strains, including the O3:K6 strain, of *V. parahaemolyticus* in oyster, (Cook 2003).

Calik and others (2002) reported that treatments of 345 MPa for 30 s could reduce cells of *V. parahaemolyticus* in pure culture ($7.6×10^6$ CFU/mL) and oysters ($8.4×10^5$ CFU/g), respectively, to non-detectable levels (<10 CFU/mL or CFU/g). A similar study also reported that pressure treatments needed to be ≥ 350 MPa for 2 min at temperatures between 1 and 35 °C and ≥ 300 MPa for 2 min at 40 °C to achieve a 5-log reduction of *V. parahaemolyticus* in live oysters (Kural and others 2008).

An added benefit of using HPP for inactivating *V. parahaemolyticus* in oysters is that the process also assists in oyster shucking by destroying the adduct muscle. Thus, oysters need to be banded before the HPP treatment to prevent opening of shell during the process. He and others (2002) reported that an HPP process of 240 to 275 MPa for less than 1 min could be used for shucking Pacific oysters with minimum changes in appearance. However, the high cost of initial investment of the high-pressure system limits its application by the shellfish industry.

2.6.2.4 Irradiation process

Irradiation is another non-thermal process of live oysters that can be utilized to destroy *Vibrio* pathogens in shellfish. A study conducted by Andrews and others (2003) found that irradiation with Cobalt-60 gamma radiation at doses of 1.0-1.5 kGy was effective to reduce *V. parahaemolyticus* O3:K6 strain from 4 log units to non-detectable levels (<3.0 MPN/g) in artificially inoculated oysters. Sensory analysis, including difference tests, conducted with 146 volunteers showed that the participants could differentiate non-irradiated from irradiated oysters. The investigators concluded that low dosages of irradiation did not kill oysters or affect the sensory quality of oysters (Jakabi and others 2003). However, the reluctance among consumers to accept irradiated food limits its usage.

2.6.3 Relaying and Depuration

Relaying and depuration are processes utilizing the natural water-filtering mechanism of oysters to release contaminants from the digestive tract in clean and unpolluted water. The offshore relaying process typically moves shellfish from polluted areas to an unpolluted waterway for natural biological purification before harvesting. Motes and DePaola (1996) reported that oysters naturally contaminated with 3-4 log MPN/g of *Vibrio vulnificus* could be reduced to <10 MPN/g by being relayed to unpolluted offshore water with a salinity of 30-34 ppt for 7-17days. By extending the process to 49 days, the levels of *V. vulnificus* could be further reduced to a mean level of 0.52 MPN/g. However, oysters cultured at low salinity concentrations

might not be able to survive for such a long period in an environment containing salinity as high as 30-34 ppt.

Increased pollution along coastal lines has resulted in reduced clean area for growing shellfish. Most of the area remaining for oyster production is suffering from increased animal waste contamination due to introduction of large amounts of bacteria including coliform and potential human pathogens from farmland into the marine environment (Yeung and Boor 2004). Therefore, it has becomes more and more difficult to use the relaying process due to the lack of clean and unpolluted marine environment.

Depuration is a process of holding filter-feeding shellfish in clean seawater to allow the shellfish to release sand and bacteria upon harvest (Blogoslawski and Stewart 1983). The process has a long history as a post-harvest treatment to reduce microbial contaminants in shellfish. However, studies have shown that depuration with clean seawater at regular temperatures was not effective in reducing certain persistent bacteria including *Vibrio* spp. in shellfish because of the colonization of those bacteria in the intestinal tracts (Colwell and Liston 1960, Vasconcelos and Lee 1972). Depuration with temperatures greater than 23ºC resulted in increased populations of *Vibrio vulnificus* in oysters (Tamplin and capers 1992). In most instances, total aerobic plate counts could be reduced by one log value via the depuration process. Nevertheless, reduction of bacteria to fewer than 10^4 cells per gram of shellfish was seldom seen for some species. It has been reported that no significant differences in mean counts of naturally occurring *V. parahaemolyticus*

were observed between depurated and non-depurated oysters (Eyles and Davey, 1984). Depuration in combination with chlorine, ultraviolet light, ozone and iodophors, has been studied to increase reductions of bacteria in oysters (Fleet 1978). However, none of these treatments could effectively remove *V. parahaemolyticus* from shellfish. Depuration of experimentally contaminated blue mussels with ozonated water for 44 h resulted in only about 1.0-log reduction of *V. cholerae* and *V. parahaemolyticus* while a 3-log reduction of *E. coli* in the mussels was observed by the same treatment (Croci and others 2002).

2.7 Factors affecting depuration

2.7.1   Water temperature

Water temperature is a major factor that affects the survival and growth ability of *V. parahaemolyticus*. Wu (2007) reported that *V. parahaemolyticus* strains were able to survive but unable to grow in oysters stored at 5ºC over 10 days. However, all the strains were able to survive and grow in oysters stored at 8ºC. In another study, reductions of *V. parahaemolyticus* in Gulf oysters by depuration with artificial seawater for 48 h could be increased from 1.2 log MPN/g at room temperature to 2.1 log MPN/g at 15 ºC (Chae 2007).

In addition to its effects on the occurrence of *V. parahaemolyticus* in the marine environment, the water temperature also affects water-pumping activity of oysters. It has been reported that the pumping activity of Gulf oysters (*Crassostrea virginica*) was at correspondingly high levels when water temperatures were between

8 and 36°C (Loosanoff 1958). The pumping activity gradually decreased when water temperature dropped to 5°C. At water temperature below 3°C, the oysters would only pump very small quantities of water. Souness and Fleet (1979) studied the effects of water temperatures on depuration of Sydney rock oyster (*Crassostrea commercialis*) using a dye uptake procedure and found that the pumping rates reached a peak at 25°C and gradually reduced when water temperatures decreased to 15°C or increased to 30°C.

2.7.2 Salinity

The salinity in seawater may also affect depuration processes for reducing *V. parahaemolyticus* in oysters. Covert and Woodburn (1972) studied the effects of sodium chloride concentrations on the survival of *V. parahaemolyticus* and reported that *V. parahaemolyticus* could survive better at 48°C in tryptic soy broth (TSB) with >3% (particularly 6-9%) NaCl than in TSB without additional NaCl supplement. In addition, *V. parahaemolyticus* also survived much better in TSB with 6% NaCl than in TSB without NaCl at 5, -5 and -18°C for 9 to 16 days. *V. parahaemolyticus* also survived better in saurel extract containing 5% NaCl at -2°C for 8 days and in the extract containing 7% NaCl at -18°C for 6 days (Temmyo 1966). The salinity in the environment can also affect oyster feeding activity. Studies have reported that oyster feeding activity was greater in high salinity seawater than in low salinity environment and oysters appeared to stop feeding at the low salinities (<7.4 ppm) (Chestnut 1946, Roderick and Schneider 1994). Since oysters get used to the salinity of seawater

during growth, the National Shellfish Sanitation Program (NSSP 1990) recommended that the salinity of depuration water should not exceed 20% different than the original water where oysters are grown and harvested.

2.7.3   pH

Oysters generally have a normal water-pumping rate near pH 7.75, which is also the optimal pH for *V. parahaemolyticus* growth. The oyster's pumping activity can be affected by changes in pH values and gradually decreases when pH value in the environment dropped to 6.5 and lower (Loosanoff and Tommers 1947). It was reported that the water-pumping rate of oysters was reduced to 10% of its normal level when the pH in the environment dropped to 4.14. Therefore, an influx of acid swamp water or industrial pollution which can lower the pH of water into oyster-growing environments will result in decreased water-pumping activity of oysters.

2.7.4   Other factors

In addition to temperature, salinity and pH of water, turbidity and suspended solids in water also may affect oyster water-pumping rates. Loosanoff and Tommers (1948) reported that suspended solids at a level of as low as 0.1 g/L in seawater could reduce oyster pumping rates dramatically (by 60 %). The pumping rates were reduced by 80 and 96% when the suspend solids in seawater increased to 1.0 g/L and 3-4 g/L, respectively.

2.8 Viable but nonculturable state (VBNC) of *Vibrio parahaemolyticus*

Many bacteria may enter a viable but nonculturable (VBNC) state after exposure to an adverse environment, such as low temperatures, low or high salt concentrations, depletion of nutrients, and certain wavelengths of light (Colwell 2000, Wong and Wang 2004). In the VBNC state, the bacteria remain alive but in a dormant form. However, they will not grow or multiply on a common medium. Studies have demonstrated that bacteria cells in the VBNC state were still intact under microscopic examination and the cells could uptake and metabolize radioactively-labeled nutrients. (Tholozan and others 1999)

The VBNC state is reversible. Bacterial can enter the state when conditions become lethal for their continued growth. When the stress is removed, bacteria can revive and resume normal growth under a favorable growth condition. Under natural conditions, *V. parahaemolyticus* may enter a VBNC state during the winter. In the laboratory, *V. parahaemolyticus* can be induced to enter the VBNC state by starvation at low temperature (4ºC) (Jiang and Chai 1996). The VBNC cells have been shown to exhibit a stronger resistance to various stresses. Berlin and others (1999) observed that *V. parahaemolyticus* and several other *Vibrio* species in the VBNC state were highly resistant to hydrostatic pressures. Wong and Wang (2004) demonstrated that the VBNC *V. parahaemolyticus* was more tolerant to thermal inactivation, low salinity and mild acid stresses than the exponential phase cells. The researchers also reported that almost all the VBNC cells remained alive after being suspended in sterile water for 1 h, while almost all the exponential phase cells died after 1 h in sterile water.

The ability of *V. parahaemolyticus* to enter the VBNC state not only allows this pathogen to survive under certain stress conditions but also makes it difficult to detect. It is known that VBNC *V. parahaemolyticus* does not loose its virulence factors and may become virulent again once it is fully resuscitated from the VBNC state.

Coutard and others (2007) studied recovery of VBNC *V. parahaemolyticus* after exposure to cold temperature and reported that *V. parahaemolyticus* cells that entered the VBNC state for fewer than 4 days were able to undergo cell division when they were incubated at 20ºC. However, *V. parahaemolyticus* cells that had entered the VBNC state for more than 4 days required a higher temperature (37 ºC) to promote recovery.

2.8.1 Detection of viable but nonculturable *Vibrio parahaemolyticus*

2.8.1.1 Polymerase chain reaction (PCR) method

Since VBNC *V. parahaemolyticus* does not grow or multiply on a common medium, it can not be detected with the traditional cultural methods. However, molecular biology methods can be used to detect DNA that is specific to *V. parahaemolyticus* in the viable cells. Taniguchi and others (1985, 1986) identified a unique gene (*tl*) encoding a thermolabile hemolysin (TL) in *V. parahaemolyticus*. Although the TL is not considered a virulence factor of *V. parahaemolyticus*, the *tl* gene has been recognized a reliable marker for *V. parahaemolyticus* detection.

Tada and others (1992) developed a polymerase chain reaction (PCR) protocol using DNA probes for specific detection of the *tdh* and *trh* genes of pathogenic *V.*

parahaemolyticus. The procedure could detect both *tdh* and *trh* genes in 400 fg of cellular DNA derived from 100 cells. However, this PCR protocol requires enrichment as a pretreatment to detect pathogenic strains in fecal samples and can not be used to detect non-pathogenic *V. parahaemolyticus* strains. Brasher and others (1998) developed a double multiplex PCR assay enabling the simultaneous detection of *Escherichia coli*, *Salmonella typhimurium*, *V. vulnificus*, *V cholerae*, and *V. parahaemolyticus* in shellfish. This double multiplex PCR protocol was capable of detecting *V. parahaemolyticus* at levels of 10^1-10^2 cells through amplification of a 450 bp fragment of the *tl* gene. Later on, a multiplex PCR assay for detecting total and virulent strains of *V. parahaemolyticus* was developed by simultaneous amplification of *tl*, *tdh* (269 bp) and *trh* (500 bp) genes (Bej and others 1999). This PCR assay was used to analyze *tl*, *tdh* and *trh* genes in 111 *V. parahaemolyticus* strains isolated from clinical, seafood, environmental, and oyster plants. The *tl* gene was detected in all 111 isolates whereas *tdh* and *trh* genes were detected in 60 and 43 isolates, respectively. The sensitivity of this multiplex PCR for detecting all three genes was 10 - 100 CFU/gram of oyster tissue following homogenization in alkaline peptone water and incubation at 35°C for 6 h. This PCR method was used to analyze the *V. parahaemolyticus* strains causing the first European incidence of gastroenteritis in Spain (Lozano-León and others 2003). The two strains, which were KP+ and produced TDH, isolated from the outbreak were confirmed to contain *tl* and *tdh*, but not the *trh* gene.

2.8.1.2 Reverse transcriptase PCR

In a living cell that exhibits normal biological activity, messenger ribonucleic acid (mRNA) is constantly transcribed from a DNA template and carries coding information to the sites of protein synthesis. It changes rapidly in normal living bacterial cells, with most mRNA having a half-life of only a few minutes (Alifano and others 1994, Belasco 1993, Sheridan and others 1998). Therefore, mRNA plays a significant role in protein synthesis which is closely associated with growth bacterial cells. However, the mRNA is not expected to be detected in a VBNC bacterial cell because there is no growth activity of the cell under the VBNC stage. This makes the detection of mRNA a suitable means of identifying VBNC *V. parahaemolyticus*. A sample containing VBNC *V. parahaemolyticus* will produce a positive result by the traditional PCR assay targeting *tl* gene and a negative result by mRNA analysis.

The traditional analytical methods for detecting eukaryotic mRNA include Northern Blot and nuclease protection assays (Siebert and Larrick 1995). Among them, the Northern blotting is the most widely used method because it can determine the sizes of mRNA and is semiquantitative. However, the Northern Blot assay requires purified RNA and preparation of specific probes recognizing mRNAs separated by electrophoresis. The nuclease protection assay is a laboratory technique used in biochemistry and genetics to identify individual RNA molecules in a heterogeneous RNA sample. This technique can identify one or more RNA molecules of known sequence and is more sensitive than Northern blotting and is also semiquantitative (Reyes and Walker 1987). However, this method still requires preparation of

specifically labeled radioactive probes and blotted mRNA. The development of reverse transcriptase PCR (RT-PCR) allows the detection of mRNA with advantages over other methods, such as high sensitivity, rapid turn-around time, and use of total RNA instead of just poly (A)+ mRNA. A typical RT-PCR assay usually includes isolation of RNA from cells or tissue, reverse transcription of RNA to yield cDNA, mixing cDNA with specific primers, running PCR, ethidium bromide gel electrophoresis, and validation of PCR product. Compared to the conventional methods, RT-PCR is simple and can successfully detect a very low level of mRNA in clinic samples, such as the dystrophin gene, which is usually expresses as only 0.01-0.001% of total muscle mRNA, in patients with muscular dystrophy (Chelly and others 1988, Rappolee and others 1988). RT-PCR also has an advantage over other methods in detecting the poorly expressed multidrug resistance gene *mdr-1* (Fuqua and others 1990, Murphy and others 1990). Additionally, RT-PCR is a valuable tool for analyzing genes that are expressed in tissues for a very short time (minute size) (Gaudette and Crain 1991). In the last decade, RT-PCR has been used for detecting the existence of VNBC *Enterococcus faecalis* in nutrient-poor sterilized lake water (Lleo and others 2000) and monitoring VNBC populations of environmental and clinical *V. vulnificus* strains maintained in ASW (Fischer-Le Saux and others 2002).

Chapter 3

**Refrigerated Seawater Depuration for
Reducing *Vibrio parahaemolyticus* Contamination
in Raw Pacific Oyster (*Crassostrea gigas*)**

3.1 Abstract

This study investigated the effectiveness of refrigerated seawater (5°C) depuration for reducing *V. parahaemolyticus* in raw Pacific oysters (*Crassostrea gigas*). Raw Pacific oysters were inoculated with a mixed culture of five clinical strains of *V. parahaemolyticus* (10^{5-6} MPN/g) and depurated in refrigerated seawater (5°C) in a laboratory-scale re-circulating system equipped with a 15W Gamma UV sterilizer. Populations of *V. parahaemolyticus* in oysters harvested in winter were reduced by >3.0 log MPN/g after 96 h. The process also could reduce *E. coli* in oysters from 10^3 MPN/g to <20 MPN/g. However, the process was less efficient in reducing *V. parahaemolyticus* in oysters harvested in summer and required 144 h to achieve a 3-log (MPN/g) reduction. Analyses of reverse transcriptase polymerase chain reaction (RT-PCR) and multiplex PCR targeting RNA and DNA of *V. parahaemolyticus* revealed that no viable but nonculturable cells of *V. parahaemolyticus* existed in the oysters after the depuration. Depuration of oysters at 5ºC for up to 144 h can be used as a post-harvest process to reduce contamination of *V. parahaemolyticus* and fecal coliform in Pacific oysters without noticeable oyster fatality.

3.2 Introduction

Vibrio parahaemolyticus is the leading cause of human gastroenteritis associated with seafood consumption in the United States (Kaysner and DePaola 2001). This bacterium is frequently isolated from a variety of raw seafoods, including codfish, sardine, mackerel, flounder, clam, octopus, shrimp, crab, lobster, crawfish, scallop and oyster (Liston 1990). Consumption of raw or undercooked seafood, particularly shellfish, contaminated with *V. parahaemolyticus* may lead to the development of acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, abdominal cramps and low fever.

Numerous outbreaks of *V. parahaemolyticus* infection linked to raw oyster consumption have been documented in the United States in the past ten years (CDC 1998, 1999). This bacterium was the most frequently isolated species (51%) from 240 patients suffering from *Vibrio* infections in 2004. Among those patients infected with *V. parahaemolyticus*, 20% were hospitalized and 1% died (CDC 2004, 2006). The Centers for Disease Control and Prevention estimated a 78% increase in the incidence of *Vibrio*-associated infections in 2006. The incidence of *Vibrio* infections has increased to the highest level since FoodNet began conducting surveillances (CDC, 2006), despite efforts directed at seafood consumers (especially high-risk consumers) to warn them of the potential hazards of eating raw shellfish. The often perceived and occasionally very real threat of *V. parahaemolyticus* infection following consumption of raw or undercooked oysters is a major concern for public health and causes substantial economic losses to the shellfish industry.

Vibrio parahaemolyticus can multiply quickly in oysters to an infectious dose upon exposure to elevated temperatures and prior to consumption of the oysters (FDA 2001, Gooch and others 2002). In the process of intertidal harvest, the exposure of oysters to ambient air for several hours allows *V. parahaemolyticus* to proliferate rapidly in oysters, especially on a warm day (Nordstrom and others 2004). To minimize the risk of *V. parahaemolyticus* infections associated with shellfish consumption, shellfish harvest areas in the United States that were previously implicated in *V. parahaemolyticus* outbreaks are routinely monitored by state shellfish-control agencies to control transmission of these illnesses. However, oyster samples examined by the Washington Department of Health between May and September of 2006 found that the levels of virulent (*tdh+*) *V. parahaemolyticus* in most oysters from growing areas that were recently implicated in outbreaks were less than 50 MPN/g (personal communication). This indicates that the routine shellfish-monitoring program can not totally prevent the illness associated with raw oyster consumption. Cost-effective post-harvest processing techniques for decontaminating *V. parahaemolyticus* need to be developed.

Several processes have been reported to be capable of reducing *V. parahaemolyticus* in oysters post harvest. However, most of them require either a significant amount of initial investment or operation costs, and oysters are often killed during the process. Cost-effective post-harvest processing for reducing *V. parahaemolyticus* in raw oysters without significant adverse effects on oysters remains to be developed.

Depuration is a process of holding filter-feeding shellfish in clean seawater to allow the shellfish to release sand and bacteria (Blogoslawski and Stewart 1983). Though the process has a long history as a post-harvest treatment to reduce microbial contaminants in shellfish, it is not effective in reducing *Vibrio* contamination in oyster when conducted at room temperature (Colwell and Liston 1960, Vasconcelos and Lee 1972). Since occurrence of *V. parahaemolyticus* in the marine environments is known to be positively correlated with water temperatures (Kaneko and Colwell 1973, DePaola and others 1990, Duan and Su 2005), reducing water temperature for depuration might increase the efficacy of the process in decontaminating *V. parahaemolyticus* in shellfish. This study was conducted to determine the efficacy of refrigerated seawater depuration in reducing *V. parahaemolyticus* in raw Pacific oysters.

3.3 Materials and Methods

3.3.1 Oyster movement in refrigerated seawater

A GIII Research Gape Ometer (Pacific Shellfish Institute, Olympia, Wash.) was utilized to measure oyster's movement in a low-temperature (4ºC) environment. Eight oysters were attached to 4 rectangular boards (2 oysters per board) connected to an electronic measuring unit. A magnetic sensor was glued to the upper shell of each oyster. The boards with oysters were placed in a plastic tank containing artificial seawater (ASW) and held inside a cold room (4ºC) for 144 h. The ASW was prepared by dissolving Instant Ocean Salt (Aquatic Eco-System, Inc., Apopka, Fla.) in

deionized water according to the manufacturer's instructions. Movement of oysters in refrigerated water were measured by the Gape Ometer and recorded every 5 minutes by a computer.

3.3.2 Bacteria culture enrichment

3.3.2.1 *Vibrio parahaemolyticus*

Five clinical strains of *V. parahaemolyticus* (10290, 10292, 10293, BE 98-2029, and 027-1c1) obtained from the collection of the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, Wash.) were used in this study. Each culture was individually grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Spark, Md.) containing 1.5% NaCl (TSB-Salt) at 37°C for 18 to 24 h. Enriched cultures were streaked to individual plates of tryptic soy agar (TSA; Difco, Becton Dickinson) containing 1.5% NaCl (TSA-Salt) and incubated at 37°C for 18 to 24 h. After incubation, a single colony was picked from a plate and transferred to a tube of TSB-salt broth for incubation at 37°C for 4 h. The enriched cultures of *V. parahaemolyticus* were pooled into a sterile 50-mL centrifuge tube and harvested by centrifugation at 3,000×g (Sorvall RC-5B, Kendro Laboratory Products, Newtown, Conn.) at 5°C for 15 min. Pelleted cells were resuspended in 50 mL of sterile salt solution (2%) to produce a culture cocktail of approximately 10^{8-9} CFU/mL.

3.3.2.2 Fecal coliform

For efficacy of depuration in reducing fecal coliform contamination in oysters, an *Escherichia coli* strain isolated from the Columbia River was used. The strain was stored in a Cryobank tube (Copan Diagnostics Inc., Corona, Calif.) at -70ºC. The frozen culture was enriched in TSB at 37°C overnight (12-16 h) and the enriched culture was transferred to a fresh TSB for incubation at 37°C for 6 h to produce a culture broth of approximately 10^8 CFU/mL.

3.3.3 Oyster preparation

Raw Pacific oysters were obtained from Oregon Oyster Farm (Yaquina Bay, Newport, Oregon) and delivered in a cooler with ice to the laboratory on the day of harvest. The oysters were washed with tap water to remove mud on the shell and placed in a rectangular high-density polyethlylene (HDPE) tank (18 by 12 by 12 in; Nalgene, Rochester, N.Y.) containing artificial seawater (salinity: 34 ppt) at room temperature for 2 to 4 h before being inoculated with *V. parahaemolyticus*. Instant Algae marine microalgae concentrate (Shellfish Diet 1800, Reed Mariculture Inc., Calif.) was added to the ASW according to the manufacturer's recommendations to help oysters regain normal activities during holding in the ASW.

3.3.4 Inoculation of oysters with *V. parahaemolyticus*

For *V. parahaemolyticus* inoculation in oysters, 60 oysters were transferred from the ASW containing marine microalgae to another HDPE tank of 30L fresh

ASW containing *V. parahaemolyticus* culture cocktail at a level of approximately 10^{4-5} CFU/mL and Shellfish Diet 1800. The inoculation was conducted at room temperature overnight (16 to 18 h) with water circulated at a flow rate of 15 L/h. Air was pumped into the solution to keep dissolved oxygen (DO) levels favorable for oyster pumping and uptake of *Vibrio*. To prepare oysters contaminated with fecal coliform for experiments, the same inoculation process was used and the prepared ASW contained *E. coli* at a level of 10^{2-3} CFU/mL. Populations of *V. parahaemolyticus* and *E. coli* in oysters were analyzed by the three-tube and five-tube most-probable-number (MPN) method (USFDA 2001), respectively, before and after the inoculation.

3.3.5 Oyster depuration

Inoculated oysters were depurated with 60 L of filtered seawater in an laboratory-scale re-circulating (25L/min) system (Figure 3.1) equipped with a 15 W Gamma UV sterilizer (Current-USA Inc., Vista, Calif.), and a water chiller (Delta Star, Aqua Logic, Inc., San Diego, Calif.) at 5°C. *V. parahaemolyticus* levels in oysters were analyzed at 0, 12, 24, 36, 48, 60, 72, 84, and 96 h of depuration for oysters harvested in the winter and at 0, 24, 48, 72, 96, 120, and 144h for oysters harvested in the summer.
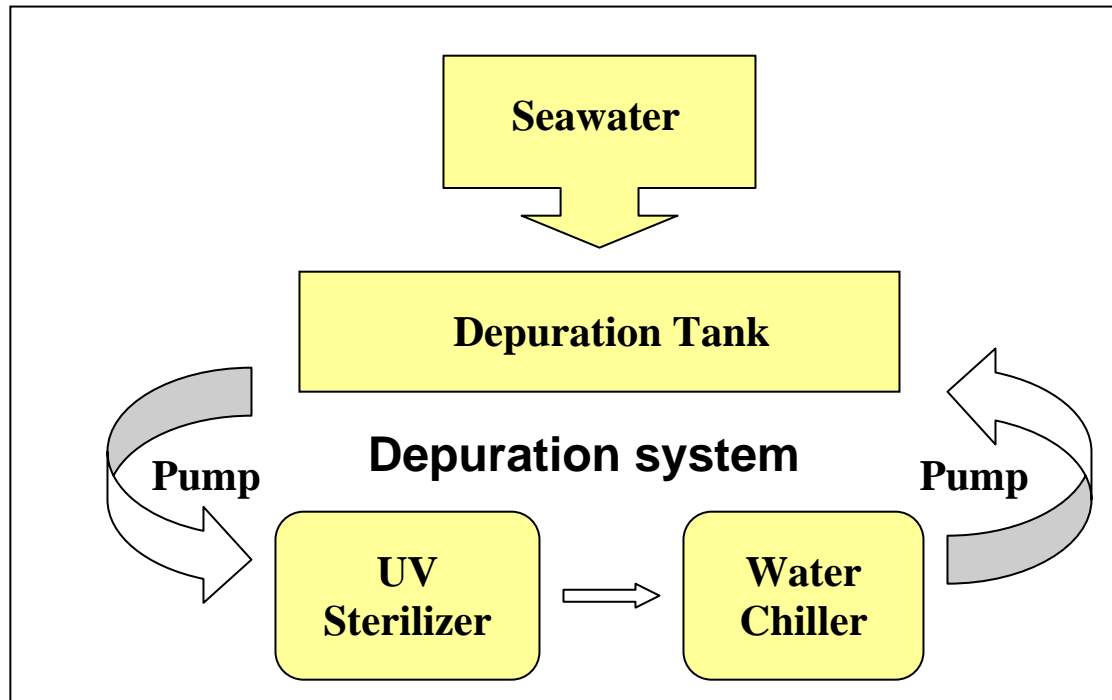
Fig 3.1 Schematic drawing of Oyster Depuration System

3.3.6 Microbiological tests

3.3.6.1 Detection of *V. parahaemolyticus*

Vibrio parahaemolyticus levels in oysters were analyzed with the three-tube MPN method described in the United States Food and Drug Administration's Bacteriological Analytical Manual (USFDA 2001). At each testing point, five oysters were randomly picked from the depuration tank, placed in a sterile stainless steel tray and shucked with a sterile shucking knife. Each shucked oyster meat was placed in a sterile blender jar and blended with 9 volumes of sterile alkaline peptone water (APW) at high speed for 1 min using a two-speed laboratory blender (Waring Laboratory, Torrington, Conn.) to prepare a 1:10 dilution sample suspension. Several additional 10-fold dilutions for each oyster sample were prepared with sterile APW. All sample

dilutions were individually inoculated into three tubes of alkaline peptone salt broth (APS). Inoculated APS tubes were incubated at 35 to 37°C for 16 to 18 h and one loopful (3mm) of enriched APS from a turbid tube was streaked onto individual thiosulfate-citrate-bile salts-sucrose agar plates (TCBS). The TCBS plates were incubated at 35 to 37°C for 18 to 24 h. Formation of colonies that are round (2- to 3-mm diameter) and green or bluish on TCBS were considered positive for *V. parahaemolyticus*. Total populations of *V. parahaemolyticus* in oysters were determined by converting the number of APS tubes that were positive for *V. parahaemolyticus* to MPN/g using an MPN table. Results were reported as the mean of five determinations.

3.3.6.2 Detection of fecal coliform

Fecal coliform in shellfish was analyzed with the five-tube MPN method described in the United States Food and Drug Administration's Bacteriological Analytical Manual (USFDA 2001). At each test point, five oysters were randomly picked from the depuration tank, placed in a sterile stainless steel tray and shucked with a sterile shucking knife. Each shucked oyster meat was placed in a sterile blender jar and blended with 9 volumes of sterile Phosphate Buffered Saline Solution (PBS, Brisbane, Calif.) at high speed for 1 min using a two-speed laboratory blender (Waring Laboratory, Torrington, Conn.) to prepare a 1:10 dilution sample suspension. Several additional 10-fold dilutions for each oyster sample were prepared with sterile PBS. The sample was first inoculated to Lauryl tryptose (LST) broth and the tubes were

incubated at 35ºC for 24 ± 2 h. After incubation, tubes that turned turbid with gas production were considered as presumptive positive for coliform. Each presumptive-positive LST tube was then inoculated into Brilliant green lactose bile (BGLB) broth and incubated at 35ºC for up to 48 h. Production of gas in the BGLB tubes confirmed presence of coliform in samples. Presence of fecal coliform in samples was determined by transferring BGLB tubes that were positive for coliform to EC broth and incubating at 44.5 ± 0.2 ºC for up to 48 h. Any EC tube that showed signs of growth with gas production was concluded to be positive for fecal coliform.

3.3.7 Statistical analysis

The log values of microbiological test results were statistically analyzed. Bacterial populations in oysters at different treatment times were analyzed with the two-sample t test (S-plus, Insightful Corp., Seattle, Wash.). Significant differences between means of treatments were determined at a level of $p \leq 0.05$.

3.3.8 Detection of VBNC *V. parahaemolyticus*

3.3.8.1 Detection of *V. parahaemolyticus* with multiplex polymerase chain reaction (PCR)

Presence of *V. parahaemolyticus* in oysters was determined with a multiplex PCR amplification procedure targeting *tl*, *tdh*, and *trh* genes according to the method of Kaysner and DePaola (2001) and Bej and others (1999). Bacterial cells in enriched APS tubes were harvested by centrifuging 1 mL of the enriched culture in a microcentrifuge tube at 15,000 × *g* for 3 min (Biofuge Fresco Analytical Instruments,

Golden Valley, Minn., U.S.A.). Pelleted cells were washed twice with 0.9% NaCl solution, resuspended in 1 mL of distilled water, and boiled for 10 min in a water bath.

The multiplex PCR amplification was conducted in a PCR reaction tube containing 4.0 μL of boiled cell suspension, 2 μM of oligonucleotide primers for each of the *tl*, *tdh*, and *trh* genes, 10 μL of a 5× PCR buffer (containing 1.25 mM MgCl$_2$), 200 μM of each of the dNTPs, 2.5 units AmpliTaq DNA polymerase (GeneAmp PCR Core Kit, Applied Biosystems, Foster City, Calif.), and 21.5 μL sterile Nanopure water (Barnstead Intl., Dubuque, Iowa) to reach a total volume of 50 μL. The oligonucleotide primers for the *tl* (L-*tl*: 5'-aaa gcg gat tat gca gaa gca ctg-3' and R-*tl*: 5'-gct act ttc tag cat ttt ctc tgc-3'), the *tdh* (L-*tdh*: 5'-gta aag gtc tct gac ttt tgg ac-3' and R-*tdh*: 5'-tgg aat aga acc ttc atc ttc acc-3') and the *trh* (L-*trh*: 5'-ttg gct tcg ata ttt tca gta tct-3' and R-*trh*: 5'-cat aac aaa cat atg ccc att tcc g-3') genes were commercially synthesized (Integrated DNA Technologies, Coralville, Iowa) according to the nucleotide sequences reported in previous studies (Taniguchi and others, 1985, Nishibuchi and Kaper, 1985, Honda and Iida, 1993). PCR amplification was performed with a Gene Cycler (Bio-Rad, Hercules, Calif.) with an initial denaturation process at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and primer extension at 72°C for 1 min. The PCR reaction tubes were kept at 72°C for 5 min after the completion of all cycles to allow final extension of incompletely synthesized DNA.

The PCR-amplified DNA (10 μL) was loaded onto a 1.5% (w/v) agarose gel and analyzed by electrophoresis conducted at 140 V for 40 min in Tris-Boric acid-

EDTA buffer (TBE buffer; Biorad Laboratories, Hercules, Calif.). After the electrophoresis, the gel was stained with ethidium bromide (1μg/mL) for 15 min followed by destaining in distilled water for 15 min (Duan and Su 2005). The DNA fragments separated in the gel were visualized by the Gel Dox^{TM} XR system (Biorad Laboratories, Hercules, Calif). Occurrence of a band of 450 bp (*tl*), 269 bp (*tdh*), or 500 bp (*trh*) on the gel indicates the presence of *tl*, *tdh*, or *trh* gene, respectively, in bacterial cells.

3.3.8.2 *V. parahaemolyticus* RNA extraction

In order to detect VBNC *V. parahaemolyticus* that did not grow in APS tubes with the MPN method, *V. parahaemolyticus* RNA was analyzed in APS tubes that yielded negative results by MPN method. The RNA extraction was conducted using PureYield^{TM} RNA Midiprep System (Promega, Corporation, Madison, Wis.). An aliquot (5 mL) of enriched APS was centrifuged at 5,500 × *g* for 5 min at 4ºC in a sterile 50-mL conical centrifuge tub (Beckman J6-MI, Beckman coulter, Fullerton, Calif.). Pelleted cells were resuspended in 1 mL of TE buffer containing lysozyme and incubated at room temperature for 5 min. The culture suspension was then mixed with 1 mL of Lysis Solution containing β-Mercaptoethanol and held in ice for 10 min to complete cell lysis. Two milliliters of the lysate was transferred to a 15-mL capped centrifuge tube and mixed with 4 mL of RNA Dilution Buffer and then 1 mL of Clearing Agent. The solution was mixed by a vortexer (Henry Troemner LLC., U.S.A), incubated at 70ºC for 5 min and allowed to cool down to room temperature.

The sample was then poured into a 50-mL collection tube containing one blue PureYield™ Clearing Column and centrifuged at 2,000 × g at 22-25ºC for 10 min. The cleared lysate was then mixed with 4 mL of isopropanol and centrifuged at 2,000 × g for 10 min in another 50-mL collection tube containing clear PureYield™ Binding Column. The Binding Column was washed twice with 20 mL of RNA Wash Solution containing ethanol. The washed Binding Column was placed in a new 50-mL collection tube followed by addition of 1 mL of nuclease-free water into the column. The centrifuge tube was incubated at room temperature for 2 min. The tube was then centrifuged at 2,000 × g for 3 min and the filtrate containing RNA product was collected.

3.3.8.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

Presence of *V. parahaemolyticus* RNA in a sample was detected with reverse transcriptase polymerase chain reaction (RT-PCR) according to procedures reported by Coutard and others (2005). Briefly, 10 μL RNA sample was mixed with 10 μL 2 × RT master mix. The 2 × RT master mix for 20 μL reaction is prepared by mixing 2.0 μL of 10 × RT Buffer, 0.8 μL of 25 × dNTP Mix (100mM), 2.0 μL of 10 × RT Random Primers, 1.0 μL of MultiScribe™ Reverse Transcriptase, and 4.2 μL of Nuclease-free H₂O (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, Calif.). The reverse transcription was performed with a Gene Cycler (Bio-Rad, Hercules, Calif.). The PCR amplification was performed at 25ºC for

10 min followed by 37ºC for 120 min and 85ºC for 5 min. The cDNA products produced from the PCR amplification were kept at 4ºC until analysis.

3.3.9 Survival of oysters after the refrigerated seawater depuration process

3.3.9.1 Room temperature storage

Oysters depurated in refrigerated seawater for 96 or 144 h were transferred to a rectangular HDPE tank holding 10 L ASW with marine microalgae at room temperature for two days. The water in HDPE tank was circulated at a flow rate of 15 L/h. Air was also pumped into the solution to keep dissolved oxygen (DO) levels favorable for oyster pumping. Oysters that opened shells in the water and closed shells upon touched were considered alive after the depuration process. Oysters that did not close shells upon touch were considered dead.

3.3.9.2 Refrigerated storage

A similar study was conducted to determine the survival of oysters at refrigerated temperature after the refrigerated seawater depuration. Oysters depurated in refrigerated seawater for 96 h were held in rectangular HDPE tanks in a cold room (5ºC) for 17 days. Oyster mortality was checked by knocking each oyster shell and recorded daily.

3.4 Results and Discussion

3.4.1 Oyster movement in refrigerated (4ºC) seawater

Oyster movement in refrigerated (4ºC) seawater as recorded by the Gape Ometer showed that Pacific oysters exhibited certain movement in the cold environment even after 6 days without nutrients. The movements of the last 4 h of day 2 (44-48 h), day 4 (92-96 h), and day 6 (140-144 h) are shown in Fig 3.2, Fig 3.3, and Fig 3.4, respectively. In the first two days, the average gaping movements were low (0.01 – 0.02 in). This is probably because that the oysters were harvested when the water temperature was around 16-17ºC; and the oysters required some time to adjust to the change of temperature to 5ºC. This was demonstrated by large gape distances (0.03 – 0.04 in) recorded for most oysters between 92 and 96 h of being held in the refrigerated seawater (Fig 3.3). The movement of oysters decreased after 6 days in the refrigerated seawater (Fig 3.4), probably because of lack of energy due to starvation. When the starved oysters were transferred to room temperature seawater containing microalgae after 6 days in refrigerated seawater, oysters which did not show obvious movement in refrigerated seawater exhibited noticeable movement (Fig 3.5). These results demonstrated that the Pacific oysters could survive a 6-day refrigerated seawater depuration without nutrient intake while exhibiting a vigorous water pumping activity during the first 92 - 96 h of the process.

3.4.2 Efficacy of refrigerated seawater (5ºC) depuration in reducing *V. parahaemolyticus* in the Pacific oysters

The efficacy of refrigerated seawater (5ºC) depuration for reducing *V. parahaemolyticus* in the Pacific oysters harvested in the winter is reported in Table 3.1. *V. parahaemolyticus* levels in the oysters were reduced by 1.25 - 1.48 log MPN/g after

24 h of the depuration process. The reductions increased to 2.28 - 2.57 log MPN/g after 48 h and to 3.09 - 3.49 log MPN/g after 96 h. These results showed that the refrigerated seawater depuration was capable of reducing *V. parahaemolyticus* in Pacific oysters by >1.2, >2.2, > 2.5 and >3.1 log MPN/g after 24, 48, 72 and 96 h, respectively. Interestingly, the refrigerated seawater (5ºC) depuration was found to be less effective in reducing *V. parahaemolyticus* in the Pacific oysters harvested in the summer (Table 3.2). While the process was able to reduce *V. parahaemolyticus* in the Pacific oysters by 1.04 - 1.31 log MPN/g after 24 h, the reductions were limited to 2.57 – 2.77 log MPN/g after 96 h. An additional 48 h of operation (6 days overall) were required to bring the reductions of *V. parahaemolyticus* to 3.00 – 3.22 log MPN/g.

Oysters are known to be capable of rapidly adjusting their biological activity in response to temperature changes (Loosanoff and Tommers 1948; Loosanoff 1958). It has been reported that each shellfish species pumps water over a certain temperature range and water-pumping activity can be affected by water temperature (Rowse and Fleet 1984; Richards 1991; Roderick and Schneider 1994). Loosanoff (1958) observed that oysters would only pump very small quantities of water when water temperature dropped to 5ºC. Therefore, shellfish normally grown in cold water tends to have an optimal depuration temperature lower than those grown in warm water. This explains why the refrigerated seawater depuration was more effective in decontaminating *V. parahaemolyticus* in the Pacific oysters harvested in the winter than in the summer.
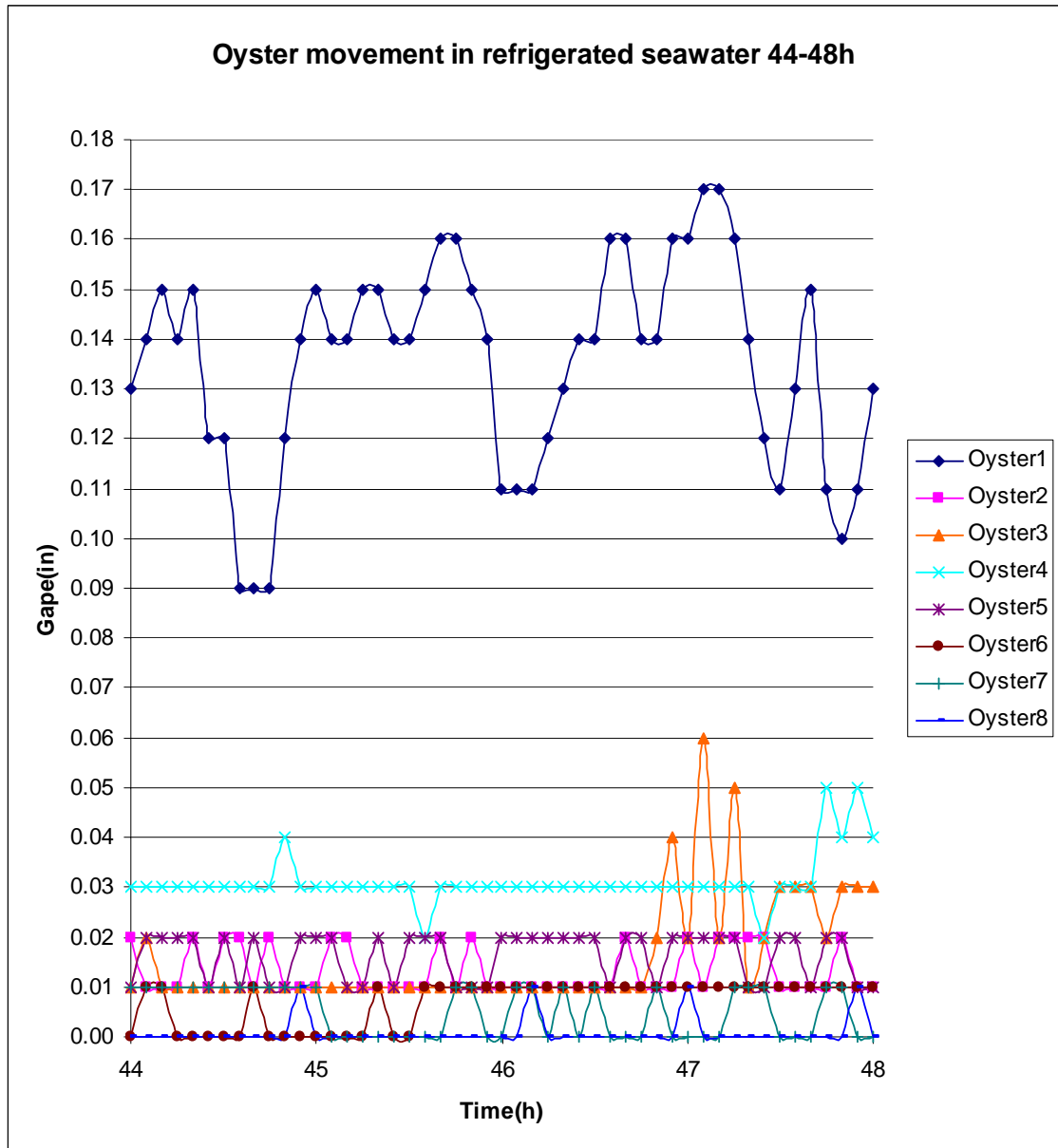
Fig 3.2 Gaping movements of oysters in refrigerated seawater (4ºC) from 44 to 48 h.
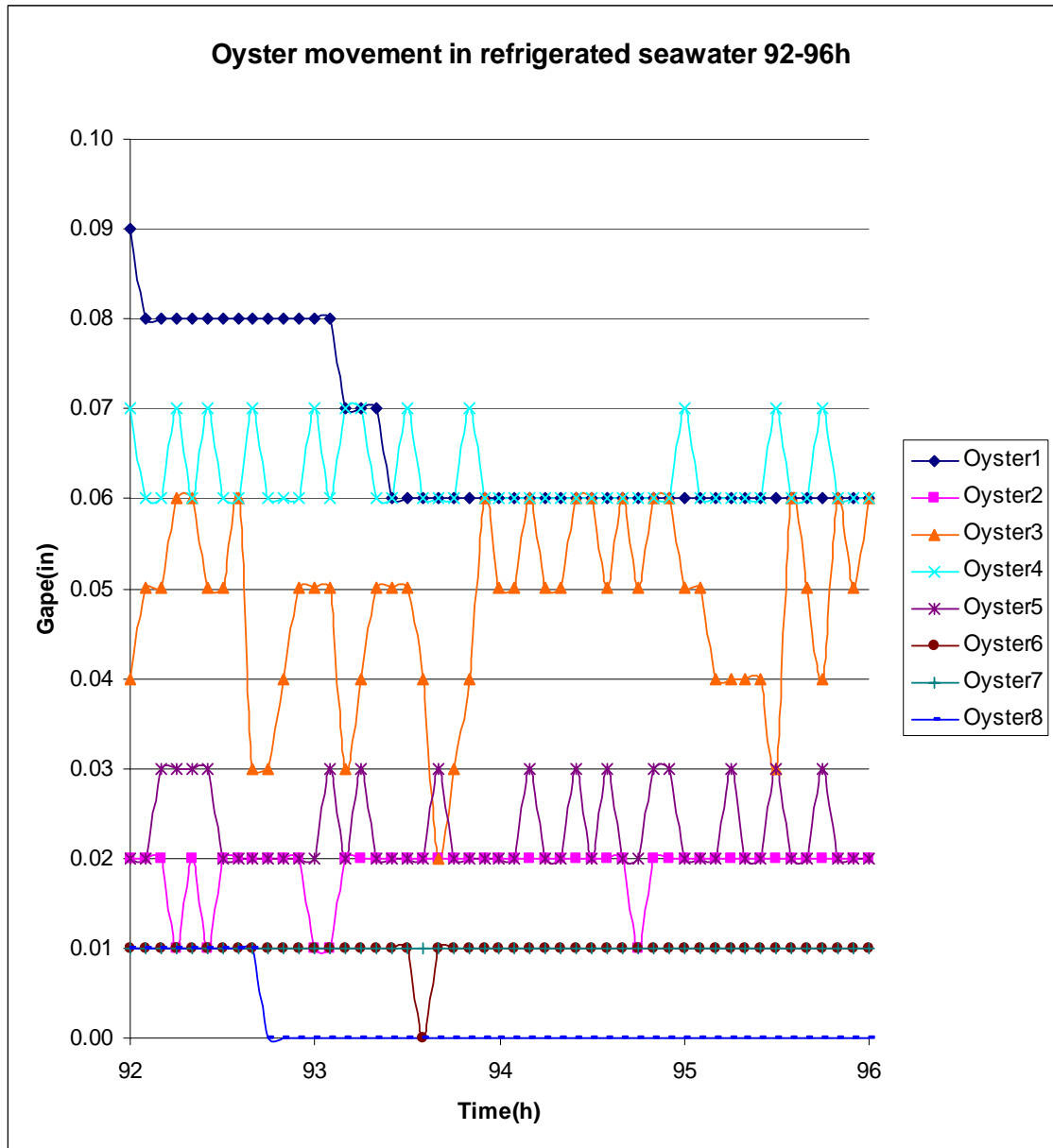
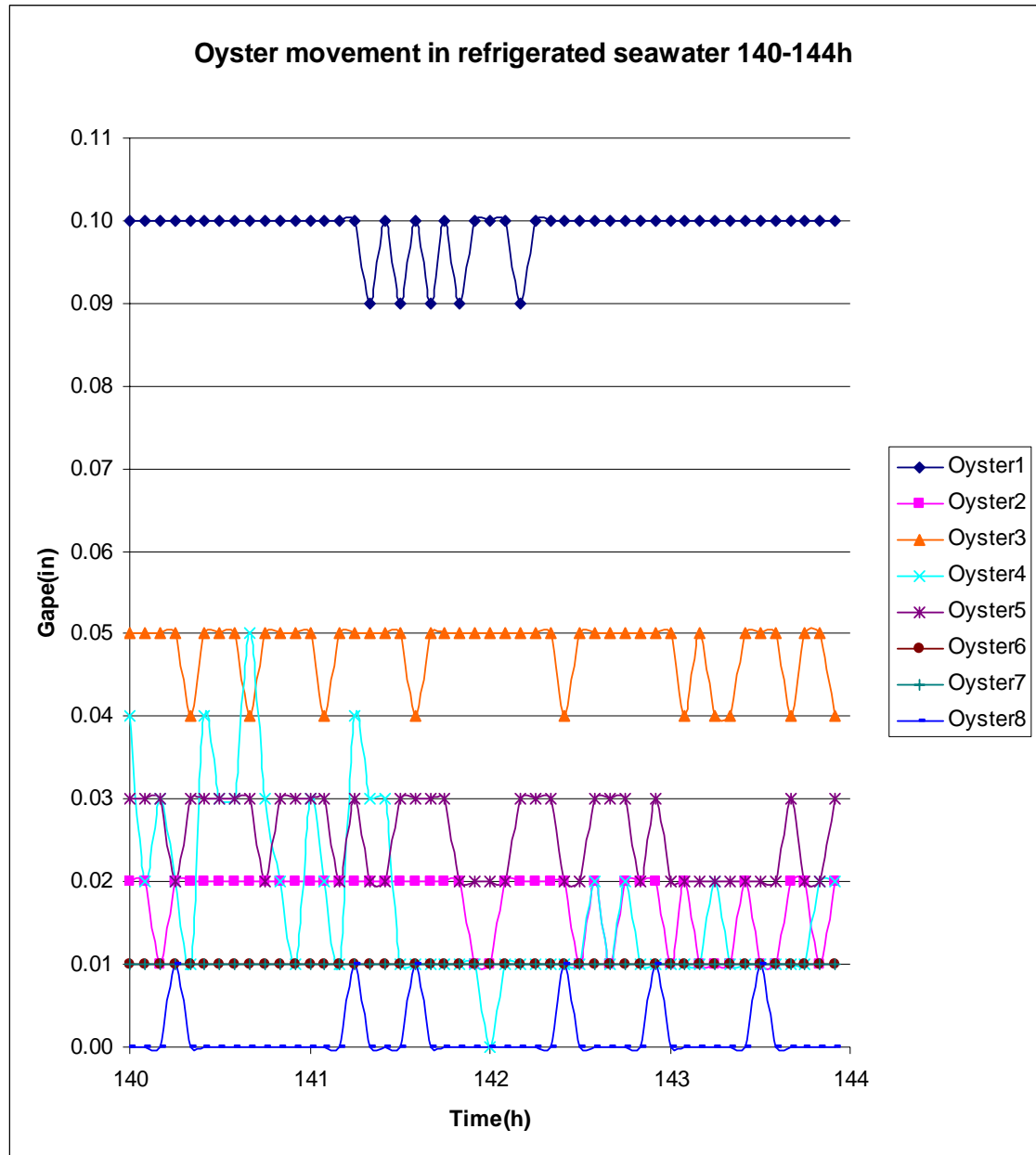Fig 3.3 Gaping movements of oysters in refrigerated seawater (4ºC) from 92 to 96 h.

Fig 3.4 Gaping movements of oysters in refrigerated seawater (4ºC) from 140 to 144 h.
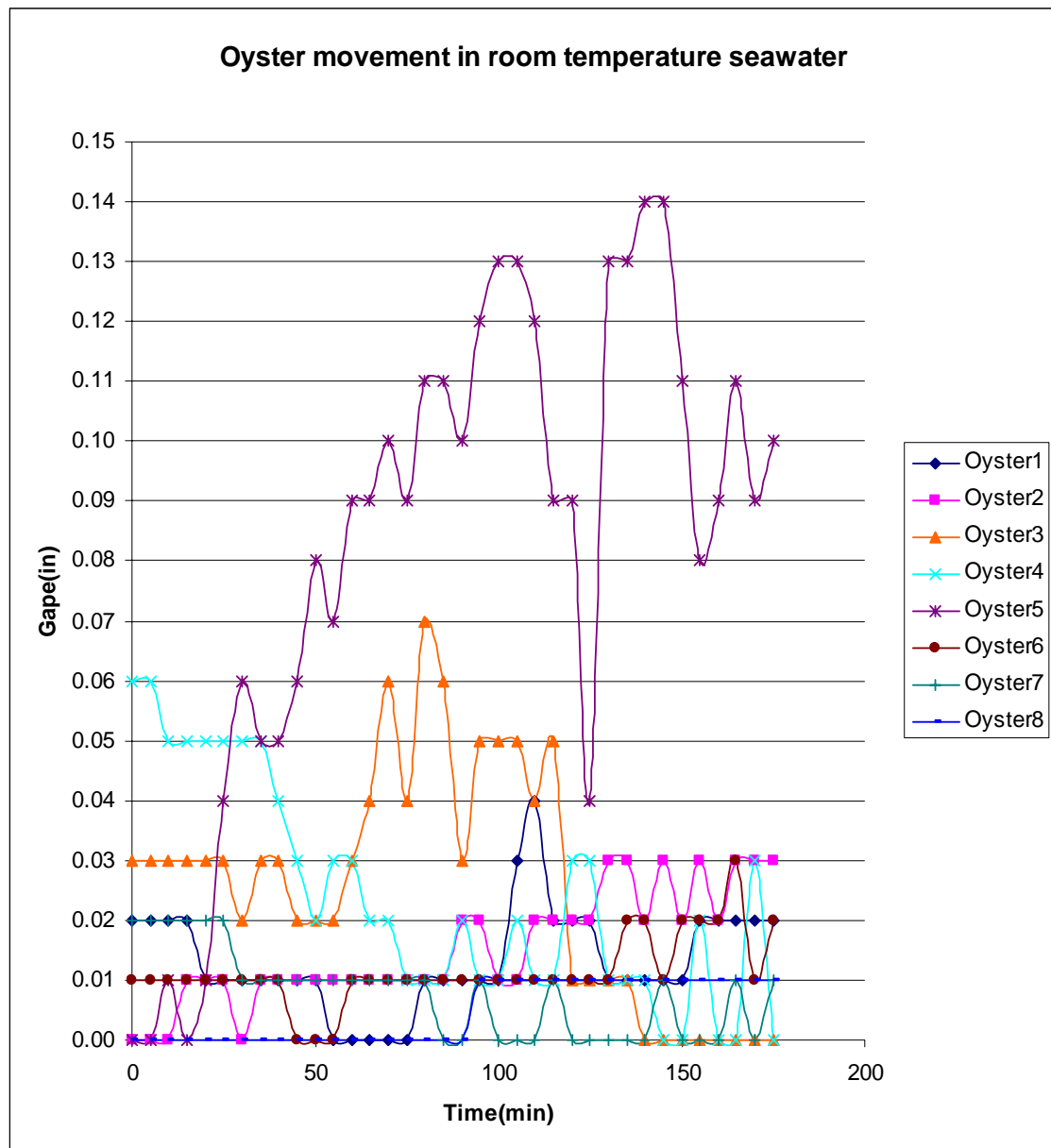
Fig 3.5 Gaping movements of oysters in seawater at room temperature after 144 h in refrigerated seawater.

Table 3.1 Efficacies of refrigerated seawater (5ºC) depuration in reducing *Vibrio parahaemolyticus* in the Pacific oysters harvested during winter time.

Time (h)	*V. parahaemolyticus* population (log MPN/g)		
	2/13/2008	2/27/2008	3/14/2008
0	5.13 ± 0.47 A[a]	4.87 ± 0.47 A	5.79 ± 0.55 A
12	4.78 ± 0.53 A (0.35[b])	4.22 ± 1.16 A (0.65)	5.57 ± 0.41 A (0.22)
24	3.70 ± 0.65 BC (1.43)	3.39 ± 1.39 AB (1.48)	4.54 ± 1.29 AB (1.25)
36	3.65 ± 0.84 BC (1.48)	2.66 ± 0.74 B (2.21)	4.49 ± 0.63 B (1.31)
48	2.86 ± 0.71 BC (2.28)	2.29 ± 1.15 BC (2.57)	3.24 ± 0.77 C (2.55)
60	2.48 ± 0.14 D (2.65)	1.44 ± 0.68 C (3.43)	2.43 ± 0.39 C (3.37)
72	2.59 ± 1.05 CD (2.54)	1.51 ± 0.37 C (3.35)	2.61 ± 0.84 C (3.18)
84	2.15 ± 0.30 D (2.98)	1.43 ± 0.47 C (3.44)	2.71 ± 0.73 C (3.09)
96	2.05 ±0.44 D (3.09)	1.38 ± 0.52 C (3.49)	2.61 ± 0.24 C (3.18)

[a] Data are means of five determination ± standard deviation. Means with the same letter in the same column have no significant difference ($p > 0.05$).
[b] Reductions in bacterial population (log MPN/g)

Table 3.2 Efficacies of refrigerated seawater (5ºC) depuration in reducing *Vibrio parahaemolyticus* in the Pacific oysters harvested during summer time.

Time (h)	*V. parahaemolyticus* population (log MPN/g)	
	7/22/2008	8/5/2008
0	6.09 ± 0.39 A[a]	5.92 ± 0.38 A
24	4.79 ± 0.37 B (1.31[b])	4.88 ± 0.61 B (1.04)
48	4.69 ± 0.85 B (1.40)	3.90 ± 0.55 C (2.02)
72	4.44 ± 0.19 B (1.65)	3.21 ± 0.39 CD (2.71)
96	3.52 ± 0.49 C (2.57)	3.15 ± 0.34 D (2.77)
120	3.34 ± 0.44 CD (2.75)	3.08 ± 0.41 D (2.84)
144	2.87 ± 0.38 D (3.22)	2.92 ± 0.31 D (3.00)

[a] Data are means of five determination ± standard deviation. Means with the same letter in the same column have no significant difference ($p > 0.05$).
[b] Reductions in bacterial population (log MPN/g)
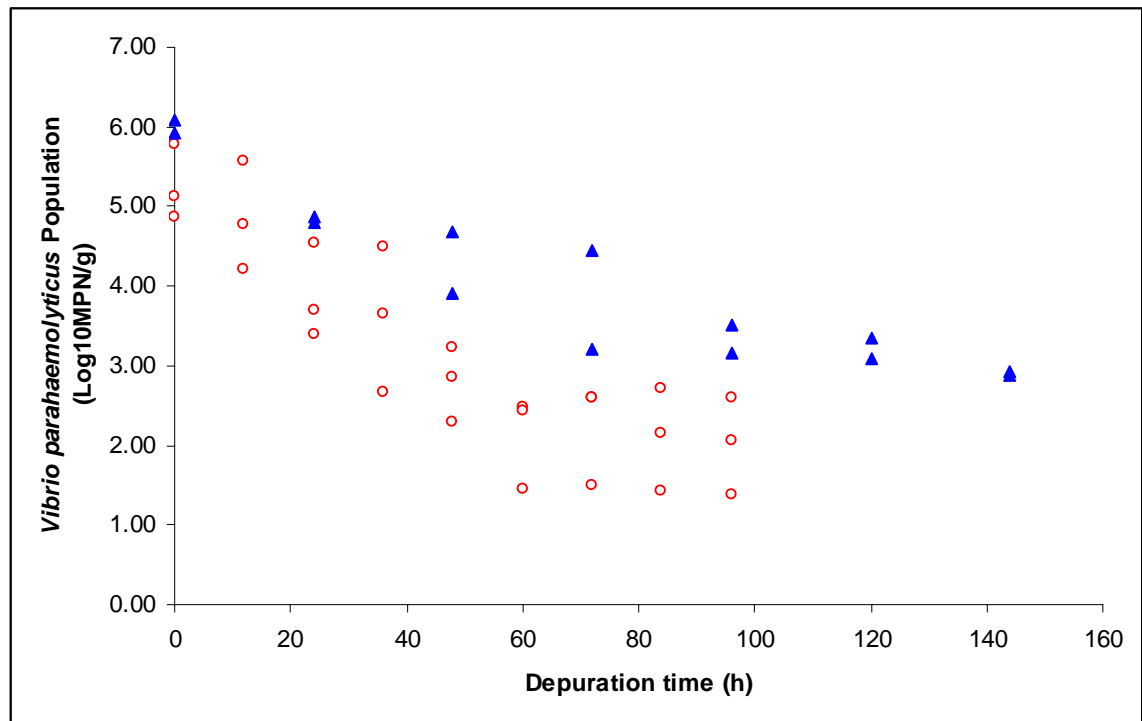
Fig 3.6 Effect of refrigerated seawater depuration on *Vibrio parahaemolyticus* levels in oysters harvested in winter and summer.

O: three depurations conducted with oysters harvested in winter months.
▲: two depurations conducted with oysters in summer months.

Table 3.3 Efficacy of refrigerated seawater depuration in reducing fecal coliform contamination in oysters.

Time (h)	Fecal coliform (log MPN/g)	
	Trial I	Trial II
0	2.79 ± 0.24 A [a]	2.96 ± 0.52 A
24	2.04 ± 0.22 B (0.93 [b])	2.66 ± 0.55 AB (0.29)
48	1.43 ± 0.78 BC (1.54)	2.09 ± 0.31 BC (0.87)
72	1.72 ± 0.33 B (1.25)	1.57 ± 0.39 CD (1.38)
96	1.14 ± 0.33 C (1.83)	1.16 ± 0.40 D (1.79)

[a] Data are means of five determination ± standard deviation. Means with the same letter in the same column have no significant difference (p > 0.05).
[b] Reductions in bacterial population (log MPN/g).

For oysters harvested in the winter, the average water temperatures of Yaquina Bay were about 7 - 9ºC (Duan and Su, 2005). Therefore, oysters only need to make a small adjustment in biological activity in response to the minor change of temperature (5ºC). On the other hand, the average water temperatures were around 16 - 17ºC at Yaquina Bay during summer months when oysters were harvested. Oysters would likely need a longer time to adjust biological activity to the new environment. Our study of oyster movement demonstrated that oysters harvested in the summer exhibited very low levels of metabolism in the first 48 h when they were held in refrigerated (4ºC) seawater (Fig 3.2). Statistical regression analysis of *V. parahaemolyticus* reductions by the refrigerated seawater depuration also showed significant differences between oysters harvested in different seasons (Fig 3.6). Greater reduction rates of *V. parahaemolyticus* were observed in oysters harvested in the winter compared to those harvested in the summer (difference in the reduction rates between two seasons estimate = 0.0151, p-value = 0.0067). In addition to the seasonal effects, depuration time was also found to be a significant factor for *V. parahaemolyticus* reduction in oysters (p-value<0.0001). This study found that *V. parahaemolyticus* could be reduced by the refrigerated seawater depuration in oysters harvested in the summer at a rate of 0.0211-log/h and in oysters harvested in the winter by a rate of 0.0362-log/h.

3.4.3 Efficacy of refrigerated seawater depuration in reducing fecal coliform contamination in oysters

The efficacy of refrigerated seawater depuration in reducing fecal coliform in the Pacific oysters is reported in Table 3.3. In two separate trials, populations of *E. coli* inoculated into oysters at levels of approximately 10^3 MPN/g were reduced to <20 MPN/g after 96 h of depuration in refrigerated seawater.

Coliforms are a group of bacteria that are gram-negative, rod-shaped, and non-spore forming organisms that ferment lactose with the production of acid and gas within 48 h incubated at 35-37°C. The United States Public Health Service adopted the enumeration of coliform as a standard of sanitary significance in 1914 (FDA 2002). However, the association of coliform bacteria with fecal contamination is not always accurate or useful because some coliforms are found naturally in the environment and can survive in water on their own (Gleeson and Gray 1996). Therefore, fecal coliform was introduced as a fecal pollution indicator. Fecal coliforms can grow in the presence of bile salts or similar surface agents, are oxidase negative, and produce acid and gas from lactose within 48 hours at 45.5°C for food testing, and 44.5°C for water, shellfish or shellfish harvest water samples (APHA 1970, 1998, Neufeld 1984, Roslev and others 2004).

Since fecal coliform can also be accumulated in shellfish through filter-feeding activity in a polluted environment, fecal coliform is regularly monitored to ensure that water bodies meet established sanitary standards for shellfish growing water. In addition, fecal coliform is also widely used for assessment of the potential presence of fecal pathogens in shellfish (West 1989).

The current National Shellfish Sanitation Program's Bacteriological Standards for the Approved Classification of Shellstock Growing Areas requires that the median or geometric mean of fecal coliform of the water sample results shall not exceed 14 MPN per 100 ml, and not more than 10 percent of the samples shall exceed 43 MPN per 100 ml for a five tube decimal dilution test (FDA 2005a).

A previous study showed that depuration of oysters in a closed recirculating system at ambient temperature could reduce fecal coliforms in oysters from 92,000 MPN/100g to less than 18 MPN/100g after 24 h (Fox and Chauvin 1988). The present study demonstrated that depuration of oysters in refrigerated seawater at 5ºC for 96 h could also reduce *E. coli* in oysters from 10^3 MPN/g to <20 MPN/g. In a closed recirculating depuration, it is necessary to sterilize the water to prevent the bacteria released from the oysters to serve as source of re-contamination during the process. A common approach is to run the circulating water through a UV sterilizer before going back to the depuration tank. In our study, the water coming out from the UV sterilizer contained no detectable levels (<1.8 MPN/mL by a five-tube MPN method) of *E. coli* (data not shown) before it was circulated back to the depuration tank. These results indicate that the refrigerated seawater depuration can be used as a post-harvest process for reducing *V. parahaemolyticus* and fecal coliform contamination in oysters.

3.4.4 Detection of Viable but nonculturable (VBNC) *V. parahaemolyticus* with reverse transcriptase PCR (RT-PCR)

To confirm the efficacy of the refrigerated seawater depuration in reducing *V. parahaemolyticus* in the Pacific oysters, reverse transcriptase PCR (RT-PCR) was

used to detect VBNC *V. parahaemolyticus* cells that might be present in oysters and could not be detected by the MPN method after the depuration process. Oysters that had been depurated in the refrigerated seawater for 96, 120, and 144 h were analyzed with the MPN method. The enriched APS cultures that yielded negative results by the MPN method were analyzed with RT-PCR and multiplex PCR targeting *tl*, *tdh* and *trh* genes.

No VBNC cells of *V. parahaemolyticus* were detected by RT-PCR or multiplex PCR in any samples that yielded negative results by the MPN method after 96, 120, and 144 h of refrigerated seawater depuration (Fig 3.7, Fig 3.8, and Fig 3.9). Multiplex PCR was capable of detecting *tl* (450 bp), *tdh* (269 bp) and *trh* (500 bp) genes as shown in Fig 3.10. The VBNC state is a complex physiological adaptation strategy that allows bacteria to survive and remain viable for extended periods of time during suboptimal growing conditions (Rahman and others 1994). Several investigators have reported that incubation of *V. parahaemolyticus* in modified Morita mineral salts solution (MMS) containing 0.5 % NaCl at 4ºC and pH 5 for 21 days could introduce formation of VBNC cells (Johnston and Brown 2002, Wong and Wang 2004). Jiang and Chai (1996) found that two Kanagawa-negative *V. parahaemolyticus* strains entered the VBNC state after 50 (strain 38C6) and 70 days (strain 38C1) when cultured in MMS at 3.5ºC. In addition, holding *V. parahaemolyticus* in ASW at 4ºC could also promote cells of *V. parahaemolyticus* to enter the VBNC state after 22 days (Coutard and other 2005).

While these studies have reported that *V. parahaemolyticus* could enter VBNC stage upon exposure of unfavorable growth conditions, particularly when exposed to low temperatures for a period of time, our study has demonstrated that a short-term (4-6 days) exposure to the refrigerated seawater at 5ºC did not induce *V. parahaemolyticus* in oysters to enter the VBNC stage.

3.4.5 Survival of oysters during refrigerated seawater depuration and at room temperature after the depuration

A total of 9 studies were conducted to determine the ability of oysters to survive the refrigerated seawater depuration. Among these studies, all the oysters were able to survive the depuration process (Table 3.4). Only in one of the 144 h experiment trials, 6 oysters died between 120 and 144 h. When the oysters were transferred to ASW at room temperature containing microalgae after the depuration process, all of them were able to actively filter water for nutrients as evidenced by shell opening (and closing upon touch) and remained alive for at least 2 days. It was not clear why the 6 oysters died after 96 h of depuration at 5ºC. A possible reason might be that the oysters were suffering from diseases or severe nutritional deficiency, which made them more sensitive to the low temperature. Nevertheless, the majority of the oysters (514 of 520) were able to survive in the cold seawater (5ºC) for up to 6 days.

Fig 3.11 shows the survival of oysters during refrigerated storage (5ºC) with and without going through 96 h of refrigerated seawater depuration. Surprisingly, oysters depurated in the refrigerated seawater survived better than those that did not

undergo by the depuration process. The survival rates of oysters during the first 7 days of refrigerated storage were similar whether the oysters had been depurated in refrigerated seawater or not. However, a sudden increase in the death rate was observed for oysters that were not depurated in refrigerated seawater after 7 days of storage in a refrigerator. The survival rate of the oysters without depuration treatment dropped from 90 % after 7 days to 44% after 9 days, while the rate for depurated oysters remained at 87% after 9 days. Even after 15 days of post-depuration storage at 5ºC, 67 % of oysters that had been depurated in refrigerated seawater remained alive in two separate trials. However, only 14% of oysters that were not depurated in refrigerated seawater were observed alive.

The rapid decline of survival rate of the oysters depurated in refrigerated seawater after 15 days of storage at refrigerated temperature might be due to severe starvation. These results indicated that the refrigerated seawater could enhance the oysters' survival in a low temperature environment. This is probably because the cold water process made the oysters more tolerant to low temperature.

In summary, refrigerated seawater depuration at 5ºC for up to 96 h can be used as a post-harvest process to reduce contamination of *V. parahaemolyticus* and fecal coliform in Pacific oysters without noticeable oyster fatality. The low-temperature depuration is a simple and economical means to reduce the risk of *V. parahaemolyticus* infection associated with raw oyster consumption and can easily be adopted by the industry to provide safe oysters for consumption.

Fig 3.7 Agarose gel electrophoresis of reverse transcriptase PCR (RT-PCR) and multiplex PCR analyses of *V. parahaemolyticus* in oysters that had been depurated in the refrigerated seawater for 96 h and yielded negative results by the MPN method.

Lanes 1 and 8: PCR molecular ruler;
Lanes 2 and 3: RT-PCR analysis of samples;
Lane 4: RT- PCR analysis of *V. parahaemolyticus* strain 10290;
Lanes 5 and 6: Multiplex PCR analysis of samples;
Lane 7: Multiplex PCR analysis of *V. parahaemolyticus* strain 10290.

Fig 3.8 Agarose gel electrophoresis of the reverse transcriptase PCR (RT-PCR) and multiplex PCR analyses of *V. parahaemolyticus* in oysters that had been depurated in the refrigerated seawater for 120 h and yielded negative results by the MPN method.

Lanes 1 and 8: PCR molecular ruler;
Lanes 2 and 3: RT-PCR analysis of samples;
Lane 4: RT- PCR analysis of *V. parahaemolyticus* strain 10290;
Lanes 5 and 6: Multiplex PCR analysis of samples;
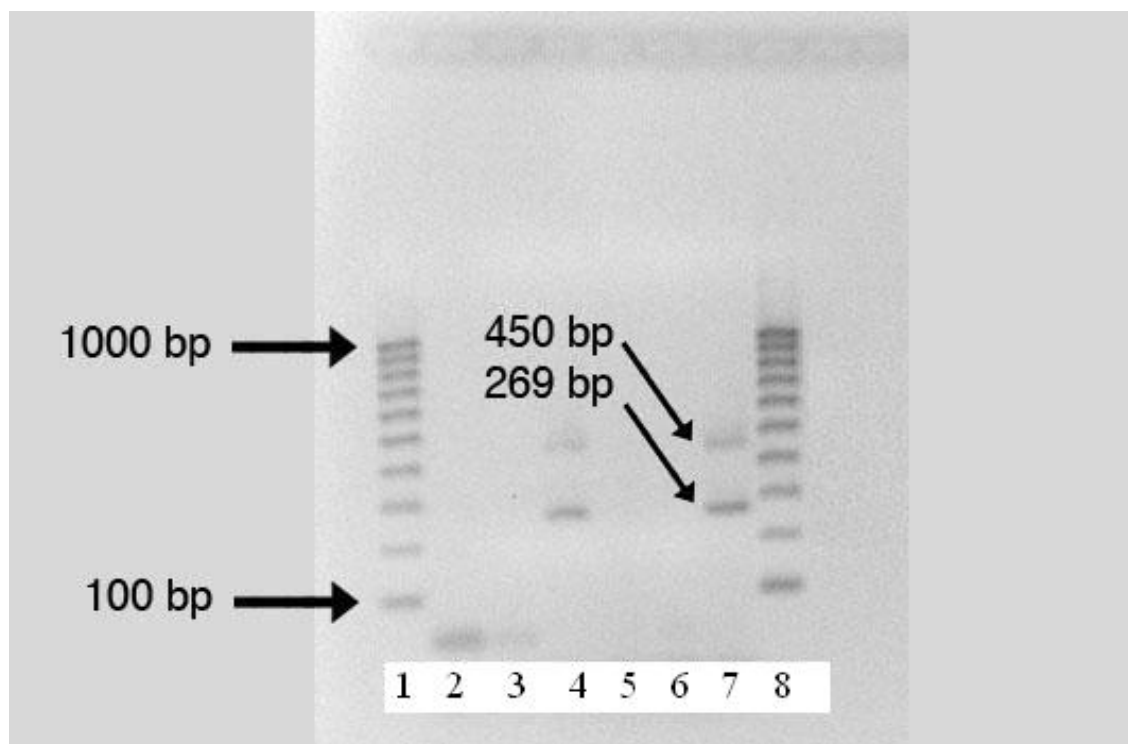Lane 7: Multiplex PCR analysis of *V. parahaemolyticus* strain 10290.
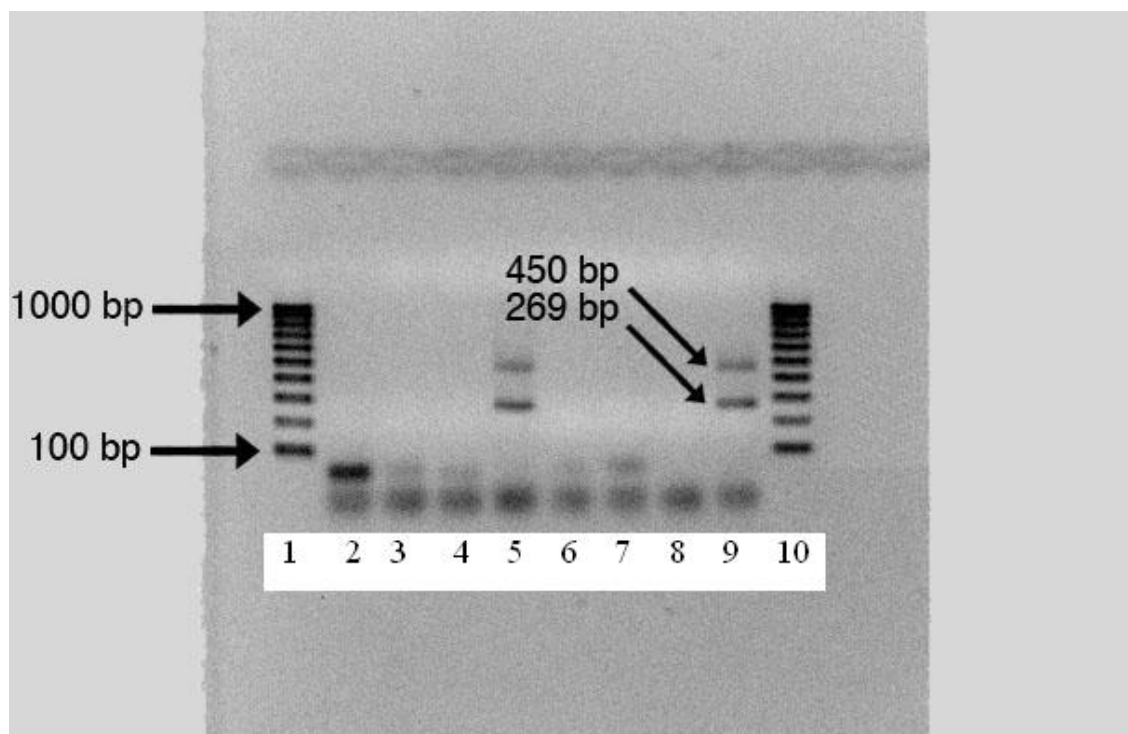
Fig 3.9 Agarose gel electrophoresis of the reverse transcriptase PCR (RT-PCR) and multiplex PCR analyses of *V. parahaemolyticus* in oysters that had been depurated in the refrigerated seawater for 144 h and yielded negative results by the MPN method.

Lanes 1 and 10: PCR molecular ruler;
Lanes 2 - 4: RT-PCR analysis of samples;
Lane 5: RT- PCR analysis of *V. parahaemolyticus* strain 10290;
Lanes 6 - 8: Multiplex PCR analysis of samples;
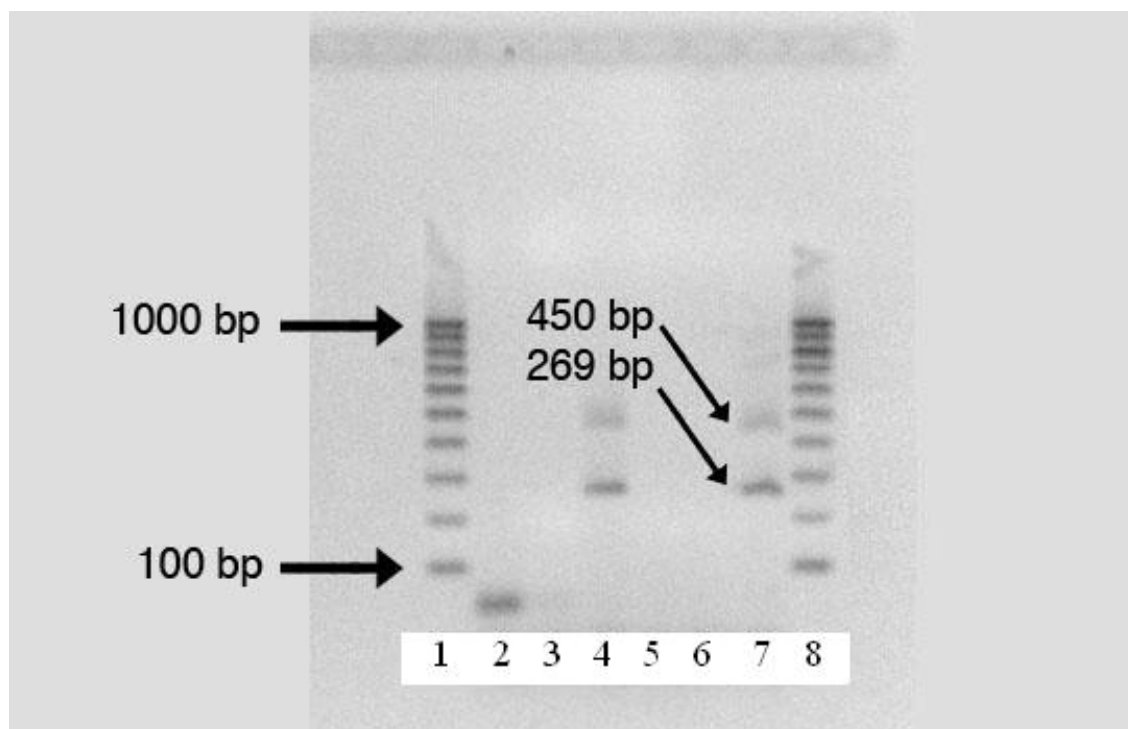Lane 9: Multiplex PCR analysis of *V. parahaemolyticus* strain 10290.
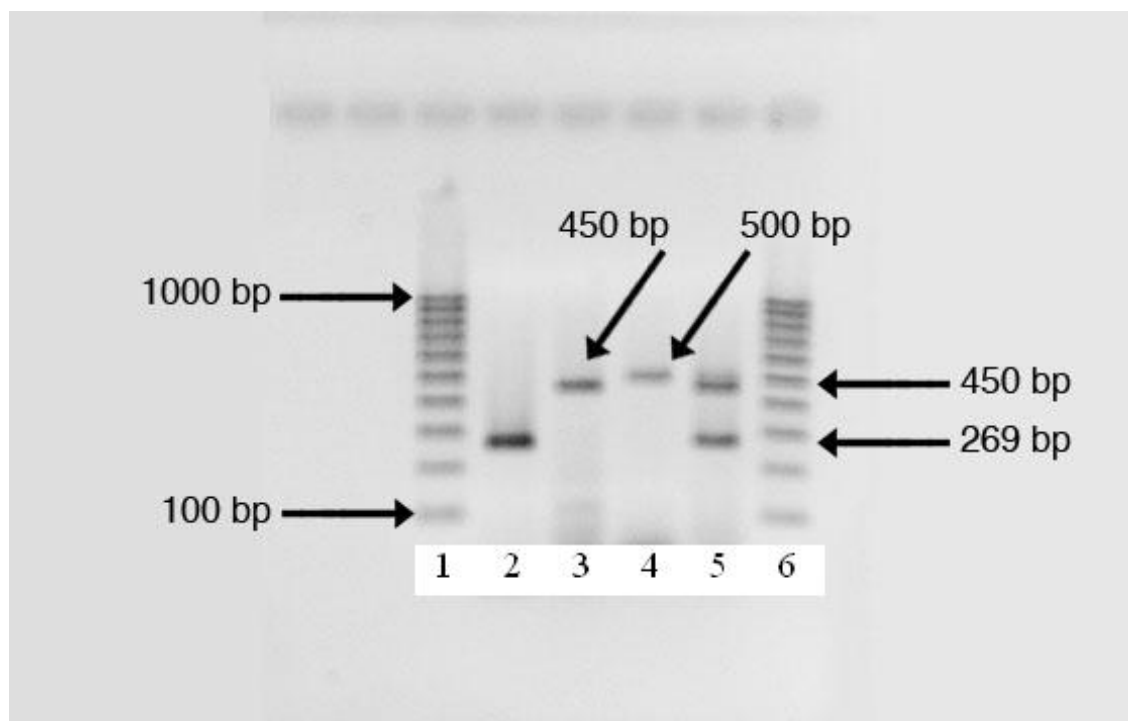
Fig 3.10 Agarose gel electrophoresis of multiplex PCR analyses of *V. parahaemolyticus* strain 10290.

Lanes 1 and 6: PCR molecular ruler;
Lanes 2: PCR amplification using oligonucleotide primers specific for the *tdh* gene;
Lane 3: PCR amplification using oligonucleotide primers specific for the *tl* gene;
Lanes 4: PCR amplification using oligonucleotide primers specific for the *trh* gene.
Lanes 5: Multiplex PCR analysis of *V. parahaemolyticus* strain 10290.

Table 3.4 Survival of oysters during refrigerated seawater (5ºC) depuration (96 – 144 h) and at room temperature after the depuration.

Trial	11/1/07	1/23/08	2/13/08	2/27/08	3/14/08	6/17/08	7/8/08	7/22/08	8/5/08
Depuration time (h)	96	96	96	96	96	144	144	144	144
Number of oysters for depuration	60	60	60	60	60	55	55	55	55
Number of oysters died during depuration	0	0	0	0	0	6*	0	0	0
Number of oysters for after depuration survival test	15	15	15	15	15	14	15	15	15
Number of oysters survived after two day beyond depuration	15	15	15	15	15	14	15	15	15

*All six oysters were alive after 120 h of the depuration process.
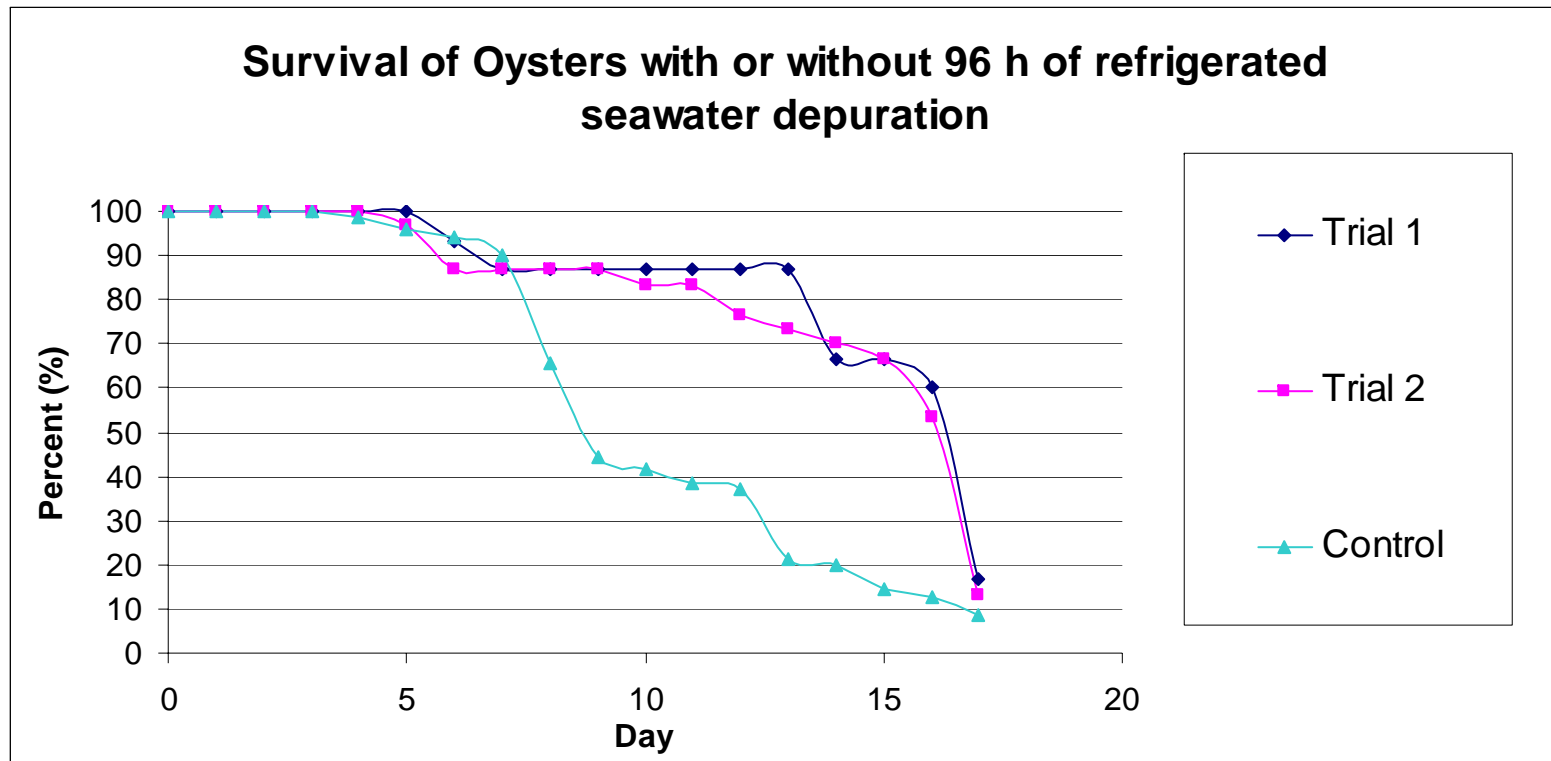
Fig 3.11 Survival of oysters during refrigerated storage (5ºC) with or without 96 h of refrigerated seawater depuration.

3.5 Conclusion

Depuration of Pacific oysters (*Crassostrea gigas*) in refrigerated seawater (5ºC) for 96 h was capable of achieving greater than 3.0 log MPN/g of reductions of *V. parahaemolyticus* in oysters harvested in the winter. Similar reductions of *V. parahaemolyticus* could also be achieved in oysters harvested in the summer. Refrigerated seawater depuration of Pacific oysters for up to 144 h did not promote *V. parahaemolyticus* to enter the VBNC stage. The process enhanced the oysters' ability to survival in subsequent storage in refrigerated temperature. Based on these results, refrigerated seawater can be used as a simple post-harvest processing technique for reducing *V. parahaemolyticus* contamination in raw oysters without apparent fatality of oysters and it will reduce the risk of *V. parahaemolyticus* infection associated with raw oyster consumption.

3.6 Acknowledgement

Chapter 4

Conclusion and Future Study

V. parahaemolyticus is recognized as the leading cause of human gastroenteritis associated with raw seafood consumption in the United States, and has caused increasing global outbreaks over the last decade (Kaysner and DePaola 2001, Su and Liu 2007). Although a number of processes, including low-temperature pasteurization, high pressure processing, and irradiation, are capable of reducing *V. parahaemolyticus* in oysters post harvest (Andrews 2004), most of them require a significant amount of initial investment or operation costs, and the oysters are often killed during processing. Cost-effective post-harvest processing for reducing *V. parahaemolyticus* in raw oysters without significant adverse effects on the oysters remains to be developed**.**

In order to remove sand and pathogen contamination, depuration has been used as a post-harvest treatment in the shellfish industry for more than 75 years (Canzonier 1991). However, in previous studies clean seawater depuration at ambient temperature did not work effectively for reducing *V. parahaemolyticus* level in oysters. The present study investigated the potential application of refrigerated seawater depuration as a post-harvest processing for reducing *V. parahaemolyticus* in raw Pacific oysters.

Our research showed that oysters were able to filter seawater at a low temperature (4°C) and survive in the refrigerated seawater (4°C) for at least 96 h without noticeable fatality. Depuration of raw Pacific oysters (*Crassostrea gigas*) in refrigerated seawater (5ºC) for 96 h was capable of achieving greater than 3 log

MPN/g of reductions of *V. parahaemolyticus* in oysters harvested in winter. However, the same process had to be carried out for 144 h to achieve 3.0 log MPN/g of reductions of *V. parahaemolyticus* in oysters harvested in summer. This is probably because of a larger gap between the refrigerated seawater and the water temperature (16 – 17°C) during summer time than in winter time (7 – 9°C) and therefore the oysters needed a longer time to acclimate to the environment of a different temperature for normal activity.

Depuration of the oysters in refrigerated seawater for either 96 or 144 hours enhanced their ability to survive during subsequent storage at refrigerated temperature (5°C). When stored at 5°C, the survival rates of oysters without the depuration treatment dropped from 90 % after 7 days to 44% after 9 days, while the rates of oysters that had been depurated in refrigerated seawater remained at 87% after 9 days. Even after 15 days of post-depuration storage at 5°C, 67 % of oysters that had been depurated in refrigerated seawater remained alive in two separate trials. However, only 14% oysters in the control groups remained alive. This may be due to increased tolerance for low temperatures developed over time.

The refrigerated seawater depuration was also capable of reducing fecal coliform from 10^3 MPN/g to less than 20 MPN/g in raw oysters. This study demonstrated that refrigerated seawater depuration was an effective, simple and economical treatment for reducing *V. parahaemolyticus* and fecal coliform contamination in raw oysters. This process can be easily adopted by the shellfish

industry for producing safe oysters for consumption and to reduce *V. parahaemolyticus* infection associated with raw oyster consumption.

Although this study reported that refrigerated seawater (5ºC) depuration could be used to decontaminate *V. parahaemolyticus* by >3.0 log MPN/g, the efficacy of the process varied among oysters harvested at different seasons. In this study, the temperature difference between water at oyster harvest area and the refrigerated seawater used in study was about 3ºC in winter and >11ºC in summer. The efficiency of the depuration in reducing *V. parahaemolyticus* in oysters harvested in winter (0.0362-log/h) was higher than in oysters harvested in summer (0.0211-log/h). Analysis of these results suggested that it might be possible to identify a stepwise cooling depuration process to aid in the acclimation of oysters to an optimal refrigerated seawater temperature. Key discoveries for the stepwise cooling process would be to determine the maximum temperature difference between each step while maintaining a high reduction rate on *V. parahaemolyticus* in oysters.

Finally, oysters may also be contaminated with other foodborne pathogens, such as enterotoxigenic *E. coli*, *Campylobacter jejuni*, *Staphylococcus aureus*, and *Salmonella*, and may serve as the vehicle for outbreaks caused by other infectious agents (Andres Mane Romero Ayulo and others 1994, Hood and others 1983, Waage and others 1999). Future research may include determination of the effectiveness of refrigerated seawater depuration on reducing those foodborne pathogens in raw oysters.

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