# AN ABSTRACT OF THE THESIS OF

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Title: Factors Affecting the Efficacy of Depuration for Decreasing *Vibrio parahaemolyticus* Contamination in Pacific Oysters (*Crassostrea gigas*).

Abstract approved:

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*Vibrio parahaemolyticus* is a foodborne pathogen recognized as the leading cause of acute gastroenteritis associated with consumption of raw and undercooked seafood, particularly raw oysters, with major symptoms of nausea, vomiting, abdominal cramps and diarrhea. It is estimated that 45,000 cases of *V. parahaemolyticus* infection occur each year in the United States. In order to reduce the high risks of *V. parahaemolyticus* infection associated with raw oyster consumption, post-harvest processes capable of decreasing *V. parahaemolyticus* levels by >3.52 log MPN/g are recommended by the U.S. Food and Drug Administration for oyster processing upon harvest.

Depuration is a process of holding shellfish in clean seawater allowing shellfish to purge contaminants and may be applied by the shellfish industry as a post-harvest process to reduce contamination of *V. parahaemolyticus* in oysters. Currently, depuration at controlled temperatures between 7 and 15°C for 5 days has been developed to achieve >3.0 but <3.52 log MPN/g reductions of *V. parahaemolyticus* in

oysters. The aim of this study was to investigate the factors, including water pH value, water temperature and oyster to water ratio, affecting the efficacy of depuration in decreasing *V. parahaemolyticus* in oysters and to improve the efficacy of depuration process to achieve >3.52 log MPN/g reductions of *V. parahaemolyticus* in oysters for application as a post-harvest treatment of oysters by the shellfish industry to produce safe oysters for raw consumption.

Studies of growth of *V. parahaemolyticus* in trypticase soy broth with 2% salt (TSB-Salt) medium of various pH values (5.5, 7.3 and 9.0) found that growth of five clinical *V. parahaemolyticus* strains were retarded in TSB-Salt at pH 5.5 compared with at pH 7.3 or 9.0. Investigation of oyster gaping in artificial seawater (ASW) of different pH values ranging from 4.0 to 10.5 unveiled that oysters survived well in ASW of pH between 5.0 and 9.5 at room temperature. Based on these findings, oyster depuration was conducted in a lab-scale depuration system with pH value of ASW being controlled at 5.5 or 7.0 at 12.5°C or 20°C for 5 days with an initial *V. parahaemolyticus* level in oysters of $10^{4-5}$ MPN/g. Depuration with ASW (pH 8.3) without control of pH value was used as a control.

Depuration in ASW of pH 5.5, 7.0 and 8.3 at 20°C for 5 days resulted in 0.7-2.0, 1.7-2.0 and 2.8 log MPN/g reductions of *V. parahaemolyticus*, respectively. Greater reductions (1.6-2.1 log MPN/g at pH 5.5, 2.9-3.0 log MPN/g at pH 7.0 and 3.5 log MPN/g at pH 8.3) of *V. parahaemolyticus* in oysters were observed after depuration with ASW at 12.5°C for 5 days. Decreasing pH value of ASW for depuration resulted in decreased efficacy of the process in reducing *V. parahaemolyticus* contamination in oysters. Study of effects of different oyster to water ratios (number of oyster :

volume of water) of 1:1, 1:1.5, 1:2, and 1:2.5 revealed that depuration with oyster to water ratio of 1:2 could achieve >3.52 log MPN/g reductions after four days.

This study improved the efficacy of depuration with ASW at 12.5°C to deliver >3.52 log MPN/g reductions of *V. parahaemolyticus* in the Pacific oysters. This controlled depuration may be applied as a post-harvest process to produce safe oysters for raw consumption. Future studies are needed to validate the efficacy of this controlled depuration for commercial application by the shellfish industry.

# Factors Affecting the Efficacy of Depuration for Decreasing *Vibrio parahaemolyticus* Contamination in Pacific Oysters (*Crassostrea gigas*)

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

Yishu Hou, Author

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# Chapter 1. Introduction

Foodborne illness is an important public health concern. The United States Centers for Disease Control and Prevention (CDC) estimates that 48 million people (1 in 6 Americans) get sick from foodborne diseases each year. Among them, around 128,000 are hospitalized and 3,000 die of various types of foodborne diseases (CDC, 2014).

*Vibrio parahaemolyticus* is an important seafood-borne pathogen that naturally inhabits temperate, subtropical and tropical coastal waters (Kaysner and DePaola, 2001). It is recognized as the main causative agent of human gastroenteritis disease associated with raw or undercooked seafood consumption, especially raw oyster consumption (Su & Liu, 2007; Hara-Kudo et al., 2003; CDC, 1998, 1999, 2013). The most common symptoms associated with *V. parahaemolyticus* infections are acute gastroenteritis with diarrhea, headache, vomiting, nausea, abdominal cramps and occasionally bloody diarrhea and low fever (Parveen et al., 2008). To people who have weak immune systems or chronic liver disease, *V. parahaemolyticus* may bring higher risks by leading to development of serious and deadly septicemia (Parveen et al, 2008; Rippey, 1994; FDA, 2012)

In the United States, *V. parahaemolyticus* is the most common cause of foodborne illness linked to seafood consumption with approximately 45,000 cases of *V. parahaemolyticus* infection occurring each year (FDA, 2012). More than 27 million pounds of oysters are harvested in the U.S. each year and most of them are sold alive

or shucked without further processing (Hardesty, 2001). In the late 19th and early 20th century, numerous outbreaks of typhoid fever were reported associated with consumption of raw oysters contaminated by sewage pollution (Potasman et al., 2002). Between 1997 and 2006, a total of 3,406 cases of foodborne *Vibrio* infections were reported in the U.S. Among these cases, almost all (94.5%) the patients reported having seafood one week before the illness and 1,931 (56.7%) cases were caused by *V. parahaemolyticus* with most of the cases being associated with raw or undercooked oysters consumption (Iwamoto et al., 2010). An investigation of 82 *V. parahaemolyticus* illnesses reported in 2013 revealed that 75 (91%) persons had consumed raw oysters or raw clams within 7 days before the illnesses (CDC, 2013).

*Vibrio parahaemolyticus* can be found in seawaters, sediments as well as oysters in temperate marine environments. The distribution and density of *V. parahaemolyticus* in the environments and in shellfish varies greatly depending on the seasons, geographic locations, and seawater temperature (Johnson et al., 2010). The densities of *V. parahaemolyticus* are usually higher in oysters than in seawater because the bacterium can be concentrated in oyster tissues through water-feeding activity (Parveen et al., 2008; Iwamato et al., 2010). Seawater temperature has been proved to significantly affect the distribution and density of *V. parahaemolyticus*. The seasonal distribution of *V. parahaemolyticus* in the marine environments was first reported in 1973 with increased densities of the bacterium being observed in waters, sediments and oysters when seawater temperature increased (Kaneko & Colwell, 1973, 1975). In

the Pacific Northwest area, *V. parahamaemolyticus* was mainly detected during summer months, when water temperature was above 15°C (Kaysner and DePaola, 2001). In the Chesapeake Bay of Maryland, *V. parahaemolyticus* was not detected in oysters when water temperature was lower than 9°C from November 2004 to December 2005. In addition to water temperature, other factors, such as salinity, dissolved oxygen, pH of seawater, chlorophyll *α* and plankton, may also influence the distribution and density of *V. parahaemolyticus*. (Caburlotto et al., 2010; Parveen et al., 2012; DePaola et al., 2003; Parveen et al., 2008)

Due to the high risk of *V. parahaemolyticus* infection linked to consumption of raw oysters, the United States Food and Drug Administration (FDA) initiated *V. parahaemolyticus* risk assessment to predict the occurrence of *V. parahaemolyticus* in raw oysters and established time-temperature regulations for raw oysters handling after harvest (FDA, 2011). Post-harvest processes are also suggested by FDA to reduce the level of *V. parahaemolyticus* in oysters for safe consumption. Several post-harvest processes, such as freezing and refrigeration, cooking, high pressure process, irradiation, relaying and depuration, can be utilized for decreasing *V. parahaemolyticus* levels in raw oysters.

Depuration is a controlled process by holding shellfish in clean seawater under controlled conditions to allow shellfish to purge contaminants from the digestive tract to the water over time (Blogoslawski & Stewart, 1983). It has been used as a post-harvest treatment for reducing sewage-associated bacteria, like *E. coli* in shellfish for a

long time. The efficacy of a depuration process is largely dependent on the biological activities of animals. The volume of water that has been pumped by oysters was regarded as a predictor of biological activity of oysters (Loosanoff, 1958). Optimum environmental factors, including water temperature, salinity and dissolved oxygen, are important for shellfish to effectively release the contaminants during depuration. Among them, water temperature has been regarded as a critical factor for both activity of oysters and growth of *V. parahaemolyticus*. Several depuration studies have been conducted to investigate the effect of temperature on depuration. Studies have shown that depuration with clean water at room temperature was not effective in reducing *V. parahaemolyticus* (Eyles & Davey, 1984). It was discovered that refrigerated temperature depuration (5°C) is more efficient than depuration at room temperature with >3 log reductions being observed after 4 days of duration at 5°C (Su et al., 2010). Phuvasate et al. (2012) investigated the impacts of different temperatures on the efficacy of depuration for reducing *V. parahaemolyticus* in oysters and reported that refrigerated temperature depuration between 7 and 15°C could achieve >3.0 log reductions of *V. parahaemolyticus* after 5 days of processes. Other factors, such as shellfish to water ratio, shellfish loading and water flow rate of the system may also affect the efficacy of depuration. (Lee et al., 2008) In addition, the effects of pH value of water on the efficacy of depuration for decreasing *V. parahaemolyticus* in oysters remain unknown.

In this study, we examined the survival of Pacific oysters (*Crassostrea gigas*) at various pH values and effects of pH value of water on persistence of *V. parahaemolyticus* in the oysters during depuration using UV light-disinfected seawater with various pH values (5.5, 7.0 and 8.3) at 12.5 and 20°C. The most efficient depuration process for decreasing *V. parahaemolyticus* in oysters identified from the studies was selected to investigate the effects of oyster to water ratio on the efficacy of depuration for reducing *V. parahaemolyticus* levels in Pacific oysters.

# Chapter 2. Literature Review

*Vibrio* is a genus of straight or curved Gram-negative bacteria that are ubiquitous in marine, estuarine or riverine environments (Drake et al., 2007). Among them, *Vibrio parahaemolyticus* is a human pathogen that can cause illness primarily through consumption of contaminated raw or uncooked shellfish, particularly raw oysters (Lipp & Rose, 1997; Su & Liu, 2007).

The most common symptoms associated with *V. parahaemolyticus* infections are acute gastroenteritis with occasional bloody diarrhea, diarrhea, headache, vomiting, nausea, abdominal cramps and low fever (Parveen et al., 2008). Although these symptoms are usually mild, people who have weak immune systems or chronic liver disease are at higher risk because infection by *V. parahaemolyticus* can lead to development of serious and deadly septicemia. (FDA, 2012) Human infections with *V. parahaemolyticus* have been frequently linked to consumption of raw or undercooked seafood, particularly raw oysters. (Hara-Kudo et al., 2003; CDC 1998, 1999, 2013)

*Vibrio parahaemolyticus* infection is a global concern for seafood safety. This bacterium is recognized as an important seafood-borne pathogen in the United States and throughout the world, including China, Japan, Great Britain, France and Italy (Kaysner & DePaola, 2001). *V. parahaemolyticus* was first identified as a causative agent of foodborne illness after an outbreak with 272 illnesses and 20 deaths occurred

in Japan in 1950 (Fujino, 1953). The United States Centers for Disease Control and Prevention (2013) estimates that 45,000 cases of *V. parahaemolyticus* infection occur every year in the United States. From 1967 to 1990, 14 outbreaks with 60 incidents and 159 cases of shellfish-related disease are reported correlated with *V. parahaemolyticus* in the United States (Rippey, 1994). Since 1996, increases in *Vibrio parahaemolyticus* infections have been observed worldwide (CDC, 2012). In the U.S., a total of 104 cases of a specific *Vibrio parahaemolyticus* (O4:K12) strain in 13 states in Atlantic coast areas with 6 hospitalizations were reported to CDC in 2013. Among them, 75 (91%) had raw oysters or raw clams within 7 days before their illness began (CDC, 2013).

Due to the high risk of *V. parahaemolyticus* infection transmitted by raw oysters, the United States Food and Drug Administration (FDA) initiated *V. parahaemolyticus* risk assessment to predict the occurrence of *V. parahaemolyticus* in raw oysters and established time-temperature regulations for raw oysters handling after harvest (FDA, 2011). Several post-harvest processes, such as freezing, refrigerating, high pressure processing and depuration, have been suggested and developed to enhance safety of raw oyster consumption.

## 2.1 Seafood consumption in the U.S.

Seafood is recognized as a part of healthy diet, due to its high-quality proteins, vitamins and certain essential nutrients (Domingo et al., 2006). The low-fat proteins in seafood provides long-chain omega-3 fatty acids important for early development along with eye and heart health. The low-fat diet of seafood were able to reduce the risks of Coronary Heart Disease (CHD). Seafood also contains a number of vitamins (A, B-complex, and D) and minerals (such as selenium, iodine, iron, and zinc) that have been linked to various health benefits (Hellberg et al., 2012). It has been estimated that 300 to 500 different species of fish and shellfish products are sold in the market places. Among them, ten types of fish and shellfish (Table 2.1) account for about 90% of all the seafood consumption in the U.S (Food Consumption References, 2011).

Table 2.1 Top 10 Consumed Seafoods in U.S. in 2013.

| Species or product | Amount consumed in 2013 (Pounds Per capita) | Species or product | Amount consumed in 2013 (Pounds Per capita) |
|---|---|---|---|
| Shrimp | 3.6 | Pangasius (Basa or Swai) | 0.8 |
| Salmon | 2.7 | Cod | 0.6 |
| Canned Tuna | 2.3 | Catfish | 0.6 |
| Tilapia | 1.4 | Crab | 0.4 |
| Alaska Pollock | 1.2 | Clams | 0.4 |

The daily consumption of seafood in the U.S. has increased from 60 grams per

person in 1994 to 68 grams per person in 2004. However, it has gradually decreased to 59 grams per person in 2011 (FAO, 2014). Compared with other food commodities, seafood consumption by Americans is about half the amounts of cheese consumed in the U.S.

## 2.2 Health hazards associated with shellfish consumption

Shellfish are filter-feeding animals and can accumulate environmental contaminants in the growing water. Therefore, there are health risks associated with consumption of molluscan shellfish contaminated with toxic chemicals and human pathogens, which are mainly linked to the water quality (Han et al., 1998).

### 2.2.1 Hazards associated with chemical pollutants

#### 2.2.1.1 Heavy metals

Heavy metals, such as mercury, cadmium, lead, copper, and chromium, in food products are a concern for food safety due to their chronic toxicity. Heavy metals accumulated in fish or shellfish do not necessarily affect the health of the animals, but they can be conveyed through the food chain to human and create health problems (Han et al., 1998).

Han et al. (1998) and Lee et al. (1996) have reported incidents of "green oysters" in Taiwan due to large discharges of heavy metal from acid cleaning of metal scrap in Erhjin Chi estuary area with a mass mortality of oysters being found on the oyster

beds nearby. High concentrations of copper (909 μg/g dry weight) and zinc (1293 μg/g dry weight) contributed to the green color of oysters (Han et al., 1998). The uptake of copper, zinc and arsenic by oysters are found to be significantly higher than that by other species, such as tuna, clams and shrimps. The copper and zinc concentrations detected in oysters were much higher than those detected in other types of seafood by about 48.6 and 10.7 times, respectively (Han et al., 1998).

Trace of mercury may be present in almost all kinds of fish and shellfish (Morrissey, 2004). The risk from eating fish and shellfish is generally not a health concern. However, some fish, such as tuna, sharks, swordfish, tilefish and king mackerel, may contain high levels of mercury and be harmful to unborn babies or young children's development of nervous systems (FDA, 2004). Human health risks from mercury exposure have been documented, including neurological effects, impaired fetal and infant growth, and possible contributions to cardiovascular disease. (Wang et al., 2005; Steuerwald et al., 2000) Advices have been made by the U.S. Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) for children and women who are pregnant, nursing or thinking of becoming pregnant (FDA 2004). In the U.S., the common route of mercury exposure is from consumption of seafood consumption (Chen C.Y. et al., 2008). The U.S. and Canada have established guidelines for allowable levels of mercury in fish and seafood products to be 1.0 ppm and 0.5 ppm, respectively.

#### 2.2.1.2 Persistent organic pollutants (POPs)

Persistent organic pollutants (POPs) are named due to their potential of persistence, ability of bioaccumulation and bio-magnification in the environment. Polychlorinated dibenzo-p-dioxins/dibenzofurans (PCDD/Fs) and dioxin-like polychlorinated biphenyls (dl-PCBs) are representative compounds of persistent organic pollutants (POPs), which belong to a group of structurally and chemically related halogenated aromatic hydrocarbons. There are 209 and 210 theoretical possible congeners of PCBs and PCDD/Fs, respectively (WHO 2004; Storelli, 2008). Among them, 17 PCDD/Fs and 12 PCBs congeners have been confirmed as prioritized contaminants by the World Health Organization (WHO) (WHO 2004). A short-term exposure of humans to high levels of dioxins may result in skin lesions while a long-term exposure may increase the risks of cancer and lead to toxicological symptoms, including increased cancer risks, immunotoxicity, neurobehavioural impairment, endocrine disruption, genotoxicity and birth defects. (WHO 2004; WHO 2014; Storelli et al., 2003) POPs are PCDD/Fs are usually formed as by-products during waste incineration and industrial processes, while PCBs are produced and used in dielectric fluids in transformers, capacitors and other electrical equipment (Safe, 1992). These toxic compounds can be introduced into food chain by mishandling and improper disposal of equipment containing PCBs. There is a high chance for these compounds to accumulate in the food chain, especially in animal fat, due to their high lipophilicity. Fish, especially those with higher fat levels, are more likely to be the major route of

POPs congeners into the human diet. Studies conducted in Baltic Sea showed that fatty fish, especially herring and salmon, have been found to contain high levels of PCDDs and PCBs (Leong & Gan, 2014). Another study has indicated that PCBs contamination in farmed salmon is significant higher than in wild salmon (WHO 2004).

The assessment of health risks of PCDD/Fs has been developed by the World Health Organization cooperated with the European Centre for Environment and Health (ECEH) by introducing the concept of Toxic Equivalency Factor (TEF). TEF represents the assigned individual toxic potency in relation to 2, 3, 7, 8 -TCDD, which is most toxic component and considered as the reference congener (TEF=1) (Storelli et al., 2003). The total toxic-equivalency quotient (TEQ) was then introduced to calculate the sum of the concentration of PCDD/Fs and dl-PCBs. The European Commission (EC) has introduced maximum levels for PCDD/Fs in various food products, including fishery products. The WHO/EURO (1991) recommended a Tolerable Daily Intake (TDI) for dioxin-related compounds of 10 pg TEQ per body weight per day (European Commission, 2006).

### 2.2.2 Hazards associated with toxins in shellfish

Shellfish can accumulate toxins through ingestion of algae as nutrients. Shellfish toxins (usually known as phycotoxins) are compounds of low molecular weight produced by certain algae and can cause shellfish poisoning if the contaminated

shellfish is consumed. There are five major categories of shellfish poisonings, including Neurtotoxic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP) and Ciguaterra Fish Poisoning (CFP) (Garthwaite, 2000). The Neurotoxic Shellfish Poisoning (NSP) is caused by the lipid soluble brevetoxins, leading to incoordination, paralysis and convulsions. The Diarrhetic Shellfish Poisoning (DSP) is caused by okadaic acid and analogues, with the major symptom of diarrhea. The Paralytic Shellfish Poisoning (PSP) is caused by saxitoxins (STX) produced by dinoflagellates of the genus *Alexandrium* Balech (Teegarden & Cembella, 1996). PSP is the most common and severe form of shellfish poisoning with symptoms of numbness, tingling and burning of the lips and skin, giddiness, ataxia and fever (Gessner et al., 1997). The Amnesic Shellfish Poisoning (ASP) is caused by domoic acid with symptoms of loss of balance, nausea, headache, disorientation and vomiting. In addition to shellfish, reef fish, such as greater amberjack, king mackerel, and yellowfin grouper, can also be contaminated with ciguatoxins produced by dinoflagellates and causes Ciguaterra Fish Poisoning (CFP). The symptoms of CFP are similar to but more severe than NSP toxins (Garthwaite, 2000).

### 2.2.3 Hazards associated with human pathogens

#### 2.2.3.1 Viral gastroenteritis

Norovirus is the most common cause of acute gastroenteritis in the United States and causes 19-21 million illnesses and contributes to 56,000-71,000 hospitalizations and 570-800 deaths each year (CDC, 2013). Epidemiological researches have suggested that norovirus is the primary pathogen accounted for 78% of reported shellfish-borne gastroenteritis in the U.S. in the 1990s (Burkhardt & Calci, 2000; Prato et al., 2004). Most of the infections were caused by consumption of oysters harvested from sewage-contaminated waters (Potasman et al., 2002). The shellfish-associated gastroenteritis was first found to be associated with viruses in 1977 in the United Kingdom. The infection of norovirus is generally mild and self-limiting, with symptoms of diarrhea, vomiting, nausea, abdominal pain, low fever, headache and myalgia.

Compared to norovirus, Hepatitis A virus (HAV) infection is much more serious health hazard linked to shellfish consumption, which may cause a debilitating disease and even death. A major outbreak of 300,000 cases of HAV infection was documented in Shanghai, China, in 1998, due to consumption of clams harvested from sewage-contaminated area (Tang et al., 1991). Other hepatitis viruses, like non-A or non-B hepatitis viruses have also been reported to link to shellfish consumption. (Potasman et al., 2002)

#### 2.2.3.2 Bacterial gastroenteritis

A number of bacterial agents can be associated with shellfish and cause human illness if shellfish is consumed raw or undercooked. In the late 19th century and early 20th century, numerous outbreaks of typhoid fever have been reported associated with consumption of raw oysters contaminated by sewage pollution (Potasman et al., 2002). Other bacteria, including *Salmonella spp.*, *Shigella spp.*, *Campylobacter spp.*, *Plesiomonas spp.*, *Aeromonas spp.*, *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*, have occasionally been identified as causes of gastroenteritis related to shellfish consumption (Rippey, 1994; Potasman et al., 2002). However, *Vibrio parahaemolyticus* is the human pathogen recognized the leading causes of gastroenteritis linked to seafood consumption.

## 2.3 Ecology of *Vibrio parahaemolyticus*

*Vibrio parahaemolyticus* is commonly found in marine waters, sediments and planktons in coastal areas. The distribution and density of *V. parahaemolyticus* in the environments and in shellfish vary greatly depending on the seasons, geographic locations, and seawater temperature (Johnson et al., 2010). The seasonal distribution of *V. parahaemolyticus* in the marine environments was first reported in 1973 with increased densities of the bacterium being observed in waters, sediments and oysters when seawater temperature increased (Kaneko & Colwell, 1973; Kaneko & Colwell, 1975).

Seawater temperature (usually measured as surface temperature) has been proved to be the major factor affecting the distribution and abundance of *V. parahaemolyticus*. Kaysner and DePaola (2000) reported that *V. parahaemolyticus* was detectable only during summer months in the Pacific Northwest, when water temperature was above 15°C. A nationwide survey of oysters and seawater conducted from May 1984 to April 1985 in Washington, California, Texas, Louisiana, Alabama, Florida, South Carolina, Virginia, and Rhode Island concluded that water temperature was a factor closely correlated with the *V. parahaemolyticus* density in oysters and seawater. When seawater temperature dropped below 16°C, low levels of *V. parahaemolyticus* were detected in oysters (15 cells/g) and seawaters (4 cells/ 100ml). However, the *V. parahaemolyticus* density increased to 68 cells/100ml in seawater and 160 cells/g in oysters when seawater temperature increased to around 25°C. (DePaola et al., 1990). Another study investigated the distribution of *V. parahaemolyticus* in oysters and seawater in the Chesapeake Bay of Maryland from November 2004 to October 2005 reported that *V. parahaemolyticus* was not detected in oysters when water temperature was lower than 9°C. However, 68% of oysters analyzed between April and October contained detectable levels (10 CFU/g) of *V. parahaemolyticus* when seawater temperature was higher than 14°C. A positive correlation between seawater temperature and *V. parahaemolyticus* density in the environments was also reported in two Oregon oyster-growing areas (Yaquina and Tillamook Bays) from a study between November 2002 and October 2003 (Duan and Su, 2005). The study observed

higher densities of *V. parahaemolyticus* in seawater, sediments or oyster samples in summer months, especially in July and August. Thus, seawater temperature is regarded as the main factor affecting *V. parahaemolyticus* distribution in the environments and oysters.

The relationship between *V. parahaemolyticus* density in the environments and the salinity of seawater is unclear. A few studies reported a significant relationship between *V. parahaemolyticus* density and seawater salinity (Caburlotto et al., 2010; Parveen et al., 2012; DePaola et al., 2003), while others did not (Parveen et al., 2012; Duan and Su, 2005; Johnson, 2012). A survey of *V. parahaemolyticus* levels and water salinity at three locations (Broad Creek, Eastern Bay and Chester River) in the Chesapeake Bay discovered that a lower density of *V. parahaemolyticus* was observed at a location with a lower mean salinity. However, no significant difference between the mean densities of total *V. parahaemolyticus* in oyster samples was observed among the three sites (Parveen et al., 2008).

In addition to water temperature and salinity, other environmental changes, such as ocean acidification (decrease of seawater pH), may also have an effect on *V. parahaemolyticus* distribution in the environments. The phenomena of ocean acidification are due to the accumulation of $CO_2$ dissolved in seawater. The surface ocean pH value dropped from 8.25 to 8.14 between 1751 and 2004 (Jacobson, 2005). It has been reported that ocean acidification can have a negative impact on shellfish larvae production. In the past few years, unusual ocean upwelling events generally

associated with ocean acidification occurred of the Oregon and Washington coasts and within Puget Sound resulted in heavy larval and juvenile mortalities of Pacific oysters (Feely et al., 2010)

## 2.4 Prevalence of *Vibrio parahaemolyticus* in Seafood

*Vibrio parahaemolyticus* has been isolated from a variety of seafood, including fish and shellfish, in temperate waters throughout the world. In Asia, *V. parahaemolyticus* is a common cause of foodborne disease with 496 outbreaks and 24,373 cases of *V. parahaemolyticus* infections being reported from 1996 to 1998 and 25,211 cases were recorded from 1999 to 2005. This pathogen is the most common cause of foodborne illness linked to seafood consumption in the U.S. The U.S. Centers for Disease Control and Prevention (CDC) estimated that 45,000 cases of *V. parahaemolyticus* infection occur each year in the U.S. (FDA, 2012).

*Vibrio parahaemolyticus* can be detected in seawaters, sediments as well as oysters in temperate marine areas. The densities of *V. parahaemolyticus* are usually higher in oysters than in seawater because the bacterium can be concentrated in oyster tissues through water-feeding activity (Parveen et al., 2008; Iwamato et al., 2010). Each year, more than 27 million pounds of oysters are harvested in the U.S. and most of them are sold alive or shucked without further processing (Hardesty, 2001). Between 1997 and 2006, a total of 4,755 cases of *Vibrio* illness were reported in the U.S. with 3,406 cases being food-borne infections. Among the 3,406 food-borne infection cases, almost all

(94.5%) the patients reported to have seafood one week before the illness and 1,931 (56.7%) cases were caused by *V. parahaemolyticus* with most of the cases being associated with raw or undercooked oysters consumption (Iwamoto et al., 2010).

Infections of *V. parahaemolyticus* associated with seafood other than oysters, such as Bloody clams and horse mackerel (*Trachurus japonicus*) were reported in several countries. In tropical and subtropical areas in Thailand, *V. parahaemolyticus* can be detected in seawater and seafood throughout the year (Fujino et al., 1974). A one-year survey was conducted in two area hospitals to investigate *V. parahaemolyticus* infection in Hat Yai City in South Thailand in 1999, where bloody clams consumption is popular and bloody clam was regarded as a major cause of diarrhoeal illness. Even though the epidemiological linkage between consumption of bloody clam and diarrhea illness was not clear, 38 of the 80 interviewed residents in Hat Yai city reported experiencing diarrhea after consumption of Bloody clam. In Japan, large amounts of raw seafood other than oysters were consumed. Among them horse mackerel is regarded as the target finfish of assessing the risks of *V. parahaemolyticus* infection because it is the most popular finfish harvested and consumed in Japan. In Japan, 500-800 *V. parahaemolyticus* outbreaks were generally reported affecting 10,000 people annually. Implicated foods including sashimi (responsible for 26% of outbreaks), sushi (23%), shellfish (16%) and cooked seafood (12%). (FAO/WHO, 2011)

## 2.5 Incidence of *Vibrio parahaemolyticus* infection

The history of *V. parahaemolyticus* can be traced back to the middle of twentieth century. It was first isolated in 1950 from a food poisoning outbreak related to "Shirasu" (partially boiled juvenile sardines) consumption (Kaneko & Colwell, 1973). In 1951, *V. parahaemolyticus* was first recognized as a cause of foodborne disease after an outbreak in Osaka, Japan (Daniels et al., 2000). In the outbreak, 272 people developed acute gastroenteritis and 20 died (Bubb, 1975).

*Vibrio parahaemolyticus* is a common cause for seafood-associated illnesses throughout Asia. In Japan, this pathogen was associated with over 70% of seafood poisoning cases (Sakazaki, 1979) with fish being the primary vehicle for its infection (Fujino et al., 1972). Between 1996 and 1998, 1,710 food poisoning incidents (24,373 cases) caused by *V. parahaemolyticus* were reported in Japan (IDSC, 1999). In China, 31.1% of foodborne outbreaks reported between 1991 and 2001 were caused by *V. parahaemolyticus* (Liu et al., 2004). In Taiwan, 1,495 cases of *V. parahaemolyticus* infections, accounting for 69% of all bacterial foodborne outbreaks, were reported from 1981 to 2003.

Compared to Asian countries, risks of *V. parahaemolyticus* infection are lower in Europe. Only sporadic outbreaks have been reported in some countries, such as Spain and France (Caburlotto, 2008). In Spain, 64 cases of gastroenteritis associated with consumption of raw oysters from an outdoor market in Galicia occurred between August and September 1999. Among them, *V. parahaemolyticus* was isolated from nine of the patients (Lozano-Leon et al., 2003). A serious outbreak of gastroenteritis

caused by *V. parahaemolyticus* happened in France in 1997 affecting 44 patients after consumption of shrimps imported from Asia (Robert-Pillot et al., 2004).

*V. parahaemolyticus* was first identified as an etiological pathogen in the U.S. in 1971 after three outbreaks of 425 cases of gastroenteritis in Maryland (Molenda et at., 1972). Prior to 1997, *V. parahaemolyticus* infections were rarely found outside Asia. Only 5 outbreaks (40 cases) were observed during 1993 to 1997 in the U.S. Four major outbreaks of *V. parahaemolyticus* infections involving more than 700 cases of illness associated with consumption of raw oysters occurred in 1997 and 1998 in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions of the U.S. (CDC, 1998, 1999). These include (1) an outbreak of 209 illnesses and one death associated with eating raw oysters harvested from British Columbia (BC) in Canada and from California, Oregon, and Washington in the U.S. reported from July to August 1997 (CDC, 1998) and (2) the largest seafood-associated outbreak ever recorded in the U.S. history occurred between May and July 1998 with a total of 416 people from 13 states suffering from *V. parahaemolyticus* infection after consumption of raw oysters from Galveston Bay, Texas. These outbreaks were unexpected and all clinical isolates were of a single clone (O3:K6 serotype) with an extremely high attack rate (DePaola et al., 2000).

Since then, 71 outbreaks (34 confirmed etiology and 37 suspected etiology) with 1,221 illnesses (1,056 confirmed etiology and 165 suspected etiology) and 25 hospitalizations (22 confirmed etiology and 3 suspected etiology) were reported from

1998 to 2008 (CDC, 2013), On July 16, 2004, several cases of gastroenteritis on a cruise ship were reported to the Alaska Department of Epidemiology. Among 132 passengers (70% of the 189 total passengers) that interviewed, 22 (17%) had the symptoms that match gastroenteritis. Consumption of oysters served on broad was identified as the vehicle for the illness. The attack rates for persons who ate oysters were 21% for Cruise 1 (3 persons got sick), 42% for Cruise 2 (5 persons got sick) and 27% for Cruise 3 (14 persons got sick) (CDC, 2011). The cause for the outbreak was that the oysters consumed by those patients were harvested when the average daily water temperature was above 15°C (McLaughlin et al., 2005). In 2011, 334 cases of *V. parahaemolyticus* were recorded in the U.S. with 21% of the cases being from Gulf Coast and 48% of the cases from non-Gulf Coast. An increasing number of infections of *V. parahaemolyticus* were reported since May in 2013. A total of 104 cases of *V. parahaemolyticus* infection including six hospitalizations were reported to the Centers for Disease Control and Prevention in 2013 (CDC, 2013).

### 2.6 Symptoms of *V. parahaemolyticus* infections

Gastroenteritis is the most common symptom of *V. parahaemolyticus* infections, but severe chronic diseases with relatively long duration may also be caused from the infections (Rippey, 1994). Based on the patients' information, diarrhea is the predominant symptom, followed by abdominal cramps, nausea, vomiting, fever, chills and bloody stools (Altekruse, 2000). These symptoms usually occur within 24 hours

of infection and the illness is usually self-limited. Most people suffered from the infection will recover within 3 days without visiting a hospital. However, for people with weak immune systems, liver disease, kidney disease, cancer, and AIDS, the infection may cause septicemia that is life-threatening. In addition to acute gastroenteritis, *V. parahaemolyticus* may also cause infections of the ear, eye, blood, and wounds, especially when exposed to warm seawater (FDA, 2012). A study of clinical syndromes of 206 cases of *V. parahaemolyticus* infection in Florida from 1981 to 1993 reported 120 cases (58%) of gastroenteritis, 16 cases (8%) of primary septicemia and 56 cases (27%) of wound infection (Hlady & Klontz, 1996). A clinical investigation of an outbreak occurred in Texas in 1998 revealed that 93% of the 296 residents experienced diarrhea within 24 hours of eating raw or undercooked oysters in a restaurant or an oyster bar. The incubation period ranged from 4 to 90 hours with a median value of 17 hours and a median duration of 6 days. Among over 400 patients involved in the outbreak, 15 of them were hospitalized for severe dehydration or bloody diarrhea. (Daniel et al., 2000)

## 2.7 Virulence factors of *V. parahaemolyticus*

*V. parahaemolyticus* can cause acute gastroenteritis in human. However, not all *V. parahaemolyticus* strains are pathogenic. Many factors, such as bet-hemolysis, adherence ability, enzymes and the products of the *tdh*, *trh* and *ure* genes, have been investigated to identify the pathogenicity of *V. parahaemolyticus*.

It has been found that the pathogencity of *V. parahaemolyticus* is closely related to the Kanagawa phenomenon (KP) associated with a hemolytic factor first identified in 1967 (Miyamoto et al., 1980). The hemolytic factor produces a beta-hemolysis on a special blood agar medium - Wagatsuma agar. Almost all *V. parahaemolyticus* strains isolated from clinical samples are KP positive, while only 1 to 2% of strains isolated from nonclinical sources are KP positive (Sakazaki et al., 1968; Miyamoto et al., 1969). Further study of the Kanagawa phenomenon led to the purification of a thermostable direct hemolysin (TDH) produced by *V. parahaemolyticus* and capable of producing hemolysis on the Wagatsuma agar (Honda et al., 1988). The TDH is named based on its thermostable property that its hemolytic activity cannot be inactivated by heating at 100°C for 10 min (Sakazaki et al., 1973).

Currently, methods such as PCR and DNA probe assays targeting the gene (*tdh*) encoding TDH are commonly used for detecting pathogenic strains of *V. parahaemolyticus* (Shirai et al., 1990). However, TDH is not the only virulence factor of *V. parahaemolyticus*. In 1988, Honda et al. (1988) reported an outbreak of gastroenteritis caused by KP-negative *V. parahaemolyticus*. Another virulence factor, thermostable direct hemolysin related hemolysin (TDH-related hemolysin), was discovered in clinical strains of *V. parahaemolyticus* not carrying *tdh* gene. The TDH-related hemolysin (TRH, encoded by *trh*) is immunologically related to, but not identical to TDH protein. According to the comparative analysis, there is about 68.6% similarity in nucleotide sequence between *tdh* and *trh* genes, indicating that they

evolved from a common homology ancestor (Shirain et al., 1990; Drake et al., 2007). Both *tdh* and *trh* genes have been reported in most clinical isolates from Pacific Coast in the U.S. (DePaola et al., 2003).

## 2.8 Factors affecting *V. parahaemolyticus* growth and survivals

*Vibrio parahaemolyticus* can grow at temperatures ranging from 10 to 41°C with the optimal temperature for growth between 20 and 35°C. This bacterium is halophilic and requires a minimum of 0.5% of NaCl to grow. A salt concentration of 2% is optimal for its growth (FDA, 2012). *V. parahaemolyticus* can grow over a wide pH range between 4.8 and 11.0, with an optimal range between 7.6 and 8.6 (Beuchat, 1973; Sakazaki, 1983). Liu and others (2008) reported that the best-growing condition for *V. parahaemolyticus* was at 34°C at pH 8.47 with salinity of 2.47%.

Effects of temperature, salt concentration and pH on growth of *V. parahaemolyticus* may be influenced by each other. Beuchat (1973) treated six *V. parahaemolyticus* strains with different temperatures, salt concentrations in media of different pH and reported that the tolerance of *V. parahaemolyticus* to temperature changed at with pH value changed. Beuchat (1973) investigated the relationship between temperature and minimum pH for *V. parahaemolyticus* to grow and reported that *V. parahaemolyticus* tend to growth at lower pH values as the incubation temperature increased. For example, the minimum pH for *V. parahaemolyticus* strain 107914 to grow was 7.6, 5.4, 5.2 at 9°C, 13°C and 21°C, respectively. Nishina et al.

(2004) developed a growth kinetics model to study the growth of *V. parahaemolyticus* O3:K6 under various conditions and found that the *V. parahaemolyticus* grew best at 25°C at pH 7 to 8 with salt concentration of 1% to 3%. In shrimp homogenates, *V. parahaemolyticus* could grow at pH 6.0 or higher. When adjusting the pH of shrimp homogenate was adjusted to 5.0, the population of *V. parahaemolyticus* dropped sharply, with no *V. parahaemolyticus* being detected after 15 min. While at pH 6 to 10, *V. parahaemolyticus* grew well without difference (Vanderzant & Nickelson, 1972).

## 2.9 Preventions and Controls of *Vibrio parahaemolyticus* Infection

### 2.9.1 Risks Assessment

It is estimated that one in ten American eat oysters each year. Therefore, there is a need to emphasize oyster safety controls (Altekruse, 2000). After the four *V. parahaemolyticus* outbreaks recorded in 1997 and 1998, the FDA initiated a risk assessment on controlling infection of *V. parahaemolyticus* from raw oysters. In 2005, a *V. parahaemolyticus* risk assessment model that may help to estimate the public health risk associated with the consumption of raw oysters containing pathogenic *V. parahaemolyticus* was established by the FDA (FDA, 2005). Since then, several mathematical models have been created to predict the occurrence of *V. parahaemolyticus* and assess the risk of infection due to consumption of *V. parahaemolyticus* in raw oysters. In addition, a comprehensive and up-to-date scientific framework has also been developed to help the agency to acknowledge the

progress of current programs related to *V. parahaemolyticus* in molluscan shellfish. The risk assessment also provides guidance information to predict and control the exposure of *V. parahaemolyticus* in raw molluscan shellfish, especially oysters. The exposure assessment is composed of three modules: (1) Harvest, (2) Post-harvest and (3) Consumption, which cover the whole supply chain of oysters from farms to consumers.

It is known that the distribution of *V. parahaemolyticus* in the marine environments is affected by water temperature. Consequently, levels of *V. parahaemolyticus* in oysters are influenced by the seasons and locations with different seawater temperatures. In the U.S., oysters are harvested at all seasons from four main regions: the Pacific Northwest, Mid-Atlantic, Northeast Atlantic and Gulf Coast which is divided into two regions: Gulf Coast (Louisiana) and Gulf Coast (non-Louisiana). Therefore, specific models were established for different seasons at different locations.

In addition to geographic location and harvest time, methods of harvesting also need to be considered for controls of *V. parahaemolyticus* in raw molluscan shellfish. There are two harvesting methods (dredging and intertidal) used by the oyster industry with both methods being practiced in the Pacific Northwest region. For the intertidal method, oysters are manually picked at low tide and oysters are exposed to the air for a period of time before being harvested. The exposure of oysters to warm air temperature provides an opportunity for *V. parahaemolyticus* to grow in oysters when compared with the dredging methon. Therefore, the FDA's risk assessment develops

twenty four different models concerning six oyster harvest practices in different regions [the Gulf Coast (Louisiana), the Gulf Coast (non-Louisiana), Mid-Atlantic, Northeast Atlantic and Pacific Northwest (intertidal), Pacific Northwest (Dredged) throughout the four seasons. (FDA, 2005)

### 2.9.2 Shellstock Time to Temperature Controls

The post-harvest controls of *V. parahaemolyticus* in oysters are mainly dependent on the duration from harvest to refrigeration of products. In 1997, FDA established a guidance limit of 10,000 viable cells per gram for *V. parahaemolyticus* in shellfish (ISSC, 1997). However, *V. parahaemolyticus* levels in oysters examined by the state or federal authorities were seldom higher than 1,000 cells per gram (Kaysner & DePaola, 2000). To limit growth of *V. parahaemolyticus* in contaminated oysters, the National Shellfish Sanitation Program (NSSP, 2011) Guide for the Control of Molluscan Shellfish established time-to-temperature regulations that limit the time of oysters exposed to ambient temperatures. Shellfish harvested for raw consumption need to be cooled down to 10°C (50°F) within 12, 18, 24 and 36 h after being harvested when the average monthly maximum air temperature is ≥27°C (>80°F), between 15 and 27°C (>60-80°F), between 10 and 15°C (50-60°F) and <10°C (<50°F), respectively (NSSP, 2011). In an attempt to reduce the risk of consuming raw oysters, the control authorities may temporarily close oyster harvesting area (especially in the summer time) when water temperature increase or limit the harvest period in certain

places, which depends on the water temperature. For some harvested oysters from warm waters, it is required to be labeled “For Shucking Only”.

## 2.10 Post-harvest processes

Post-harvest processes are suggested by FDA to reduce the level of *V. parahaemolyticus* in oysters. A valid post-harvest process can reduce the concentration of *V. parahaemolyticus* in shellfish by 3.52 logs and to levels <30 (NSSP, 2011), which is equivalent to reducing levels of *V. parahemolyticus* from an initial level of 100,000 MPN/gram to <30 MPN/gram. Shellfish processed by one of any approved post-harvest processes for reducing *V. parahaemolyticus* can be labeled as “PROCESSED TO ADDED SAFETY” or “PROCESSED TO REDUCE *V. parahaemolyticus* TO NON-DETECTABLE LEVELS”. Currently, several post-harvest processes, such as freezing and refrigeration, cooking, high pressure process, irradiation, relaying and depuration, can be applied to decrease *V. parahaemolyticus* levels in raw oysters.

### 2.10.1 Thermal process

Temperature is a critical factor that impacts the growth rate of *V. parahaemolyticus* in oysters. Temperature abuse is a main issue for oyster preservation by retailers. It has been reported that the population of *V.*

*parahaemolyticus* increased rapidly in live oysters when being held at 26°C (Gooch et al., 2002).

Johnson and others (1973) found that *V. parahaemolyticus* was able to survive at 4°C for at least 3 weeks with little or no apparent population change, while the growth of *V. parahaemolyticus* was detected in oysters when storing the shellfish at 35°C within 2 to 3 days. Gooch and others (2002) investigated the growth and survivial of *V. parahaemolyticus* in harvested oysters at 3°C and 26°C. *V. parahaemolyticus* population increased 1.7 log cfu/g after storage at 26°C for 10 h and 2.9 log cfu/g at 24h. However, a decrease of 0.8 log cfu/g was detected when oysters were stored at 3°C after refrigeration for 14 days. Hood and others (1983) inoculated oysters to a level of 2 log cfu/g and stored the shellstocks at 2°C, 8°C, 20°C and 35°C. *V. parahaemolyticus* population gradually decreased for the first 7 days, and became non-detectable after 14 days. Liu et al. (2009) found that a process with an ultralow flash freezing at -95.5°C for 12 min followed by 5 months storage at -21°C achieved >3.52 log reductions of *V. parahaemolyticus* in half-shell Pacific oysters.

Heat-shock process has been approved by the National Shellfish Sanitation Program (NSSP) for shellfish sellers to prepare oysters for sucking (NSSP, 2003). Andrew et al. (2000) found that low temperature pasteurization could be utilized for reducing *V. parahaemolyticus* in oysters. By placing raw oysters in 55℃ water until reaching the internal temperature of 48-50°C for 5 min, it reduced a mixture of *Vibrio vulnificus* and *V. parahaemolyticus* from >100,000 to non-detectable levels in less

than 10 min of processing. However, not all microorganisms were eliminated. There is no notable sensory difference between the pasteurized oysters and fresh raw oysters (Chen, 1996).

### 2.10.2 High pressure processing

High hydrostatic pressure processing (HHP) is a non-thermal process that has gained the interest in food industry for inactivating microorganisms in food products. High pressure processing has been used for shucking oysters for several years. It can also inactivate *V. parahaemolyticus* and *V. vulnificus* in oysters without affecting its sensory characteristics (He et al., 2000). A pressure range of 205 to 275 MPa at 10-30°C with 1-3 minutes treatments are usually used in the industries. It is reported that *V. vulnificus*, *V. parahaemolyticus* and *V. cholera* were susceptible to HPP treatment at pressure range of 200 to 300 MPa. *V. parahaemolyticus* inoculated in raw oyster homogenate at a level of 7-log cfu/g were totally inactivated after a treatment of 200 MPa for 10 min at 25°C. (Berlin et al., 1999) Kural et al. (2008) studied different pressure, time and temperature combinations for reducing *V. parahaemolyticus* contamination in oysters and observed a 5-log reduction of *V. parahaemolyticus* when oysters were processed at a pressure of 350 MPa for 2 min between temperatures of 1-35°C, or at a lower pressure of 300 MPa for 2 min at 40°C. Other than inactivating bacteria in oyster, high pressure process also helps to shucking oysters by destroying adduct muscle. A high pressure process of 293 MPa for 120 s at groundwater

temperature (8±1 °C) was validated capable of achieving greater than 3.52-log reductions of *V. parahaemolyticus* in Pacific oysters. The processed oysters had a shelf life of 6–8 days when stored at 5 °C or 16–18 days when stored in ice (Ma & Su, 2011).

### 2.10.3 Irradiation process

Irradiation is a non-thermal process that capable of inactivating bacterial pathogens in foods. Irradiation of raw oysters has a history of more than 25 years. It has been shown to effectively eliminate *Vibrio* pathogens. Andrews and others (2003) reported that *V. parahaemolyticus* O3:K6, which is regarded as the most resistant strain, was reduced to non-detectable level with 1.0-1.5 KGy exposure. *V. vulnificus* was reduced from 6-log cfu/g to non-detectable level (<3 MPN/g) after being treated with 0.75-1.25 kGy Cobalt-60 gamma radiation (Andrews et al., 2003). Mahmound and Burrage (2009) observed 6 log reductions of *V. parahaemolyticus* in pure culture, half shell and whole shell oysters after teatments with 0.75, 2 and 5 kGy, respectively, by X-ray (Mahmoud & Burrage, 2009). A sensory test with two consumer panels concluded that the taste of oysters irradiated with a dosage range of 1.0-1.5 kGy gamma rays was not significantly different from raw oysters. (Andrews et al., 2003)

On April 14, 2014, the FDA amended food additive regulations to allow the safe use of ionizing radiation for up to 6.0 kiloGray on crustaceans (e.g., crab, shrimp, lobster, crayfish, and prawns) to control foodborne pathogens and extend shelf life

(FDA, 2014). According to the FDA's amendment, use of ionizing radiation at the maximum permitted dose of 6.0 kiloGray, it will reduce (but not entirely eliminate) the number of pathogenic microorganisms in or on crustaceans. Therefore, crustaceans treated with ionizing radiation must be stored, handled, and cooked in the same way as non-irradiated foods.

### 2.10.4 Depuration

Depuration is a controlled process of holding shellfish in clean seawater to allow them to purge contaminants from the digestive tract to the water over time (Blogoslawski & Stewart, 1983). It has been used as a post-harvest treatment for reducing sewage-associated bacteria, like *E. coli* in shellfish for a long time. However, studies have shown that depuration with clean water at room temperature was not effective in reducing *V. parahaemolyticus* (Eyles & Davey, 1984). Croci et al. (2002) observed smaller reductions of *Vibrio cholerae* O1 and *V. parahaemolyticus* (approximately 1 log) than of *E. coli* (about 3 logs) after 2 days of depuration. The success of a depuration process is largely dependent on the biological activities of animals. Optimum environmental factors, including water temperature, salinity, are important for shellfish to effectively release the contaminants during depuration.

## 2.11 Factors affecting depuration

### 2.11.1 Water temperature

Water temperature is critical for both activity of oysters and growth of *V. parahaemolyticus*. The volume of oysters pump during depuration can be regarded as a predictor for releasing bacteria. The pumping rate of oysters increased rapidly from 8 to 16°C, while no further increase was observed at 16 to 28°C (Loosanoff, 1958). It has been studied that depuration with temperature higher than 23°C did not reduce *V. vulnificus* in oysters, but allowed the population of the bacteria to increase during depuration (Tamplin & Capers, 1992).

Several depuration studies have been done to investigate the effect of temperature on depuration. It is discovered that refrigerated temperature depuration (5°C) is more efficient than depuration at room temperature with >3 log reductions being observed after 4 days of duration at 5°C (Su et al., 2010). Phuvasate et al. (2012) investigated the impacts of different temperatures on the efficacy of depuration for reducing *V. parahaemolyticus* in oysters and reported that refrigerated temperature depuration between 7 and15°C could achieve >3.0 log reductions of *V. parahaemolyticus* after 5 days of processes.

### 2.11.2 Salinity

The salinity of seawater is significant for depuration of shellfish. In marine environments, the salinity of seawater is round 30 ppt. Therefore, the oysters are believed to pump water for nutrient feeding frequently at a salinity around 30 ppt but decrease pumping activity at a lower salinity. An early study reported that oysters

were suffering stress and could not be sufficiently purified when water salinity was 15-20 ppt, but were rapidly been purified at 32 to 47 ppt. (Rowse & Fleet, 1984). Phuvasate & Su (2013) studied effects of water salinity on depuration at 12.5°C and reported 2 log of reductions of *V. parahaemolyticus* in oysters were achieved after depuration using seawater with a salinity of 10 ppt for 5 day. However, >3 log reductions of *V. parahaemolyticus* in oysters were observed from depuration using seawater with salinities between 20 and 30 ppt for 5 days.

### 2.11.3 pH and other factors

The pH value of the marine environment is around pH 8.0. Changes in pH values may affect the pumping activity of oysters. Loosanoff & Tommers (1947) found that oysters could pump normally at pH 7.75, but the pumping rate dropped when pH decreased to 6.5. When pH decreased to 4.14, the oyster pumping rate was only 10% of the normal rate. Turbidity and total suspended particles can also influence oysters pumping ability and reduce the efficacy of ultraviolet light used to sterilize seawater in a depuration system. In addition, dissolved oxygen level in seawater can also affect oyster's pumping activity.

## 2.12 Objective

The impacts of temperature and water salinity on depuration for reducing *V. parahaemolyticus* in oysters have been investigated. Current knowledge reveals that

depuration at 12.5°C with seawater of 30 ppt for 5 days can deliver >3.0 log MPN/g reductions of *V. parahaemolyticus*. However, there is a lack of information regarding effects of pH value of seawater and oyster-seawater ratio on the efficacy of depuration for reducing *V. parahaemolyticus* in oysters. This study was conducted to fulfill the knowledge gap by investigating the efficacy of depuration at various pH values (5.5, 7.0 and 8.3) with different oyster-seawater ratios (one oyster per 1.0, 1.5, 2 or 2.5 L of water) for decreasing *V. parahaemolyticus* levels in contaminated Pacific oysters (*Crassostrea gigas*).

# Chapter 3. Materials and Methods

## 3.1 *Vibrio parahaemolyticus* culture

Five clinical *V. parahaemolyticus* strains [10290 (O4:K12, *tdh*$^+$ and *trh*$^+$), 10292 (O6:K18, *tdh*$^+$ and *trh*$^+$), 10293 (O1:K56, *tdh*$^+$ and *trh*$^+$), BE98-2029 (O3:K6, *tdh*$^+$ and *trh*$^-$), 1C1-O27 (O5:K15, *tdh*$^+$ and *trh*$^-$)] obtained from the culture collection of the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, WA) were used in the study.

## 3.2 Effects of pH value on growth of *Vibrio parahaemolyticus*

To determine effects of pH value on growth of pathogenic *V. parahaemolyticus*, each clinical strain of *V. parahaemolyticus* was inoculated to 10 ml trypticase soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) containing 2% NaCl (TSB-Salt) with pH values adjusted to 5.5, 7.3 or 9.0 with NaOH (0.1 N) or HCl (0.1 N) at a level of $10^{2\text{-}3}$ CFU/ml. Inoculated TSB-Salt was incubated at 35-37°C for 8 hours. Changes of *V. parahaemolyticus* populations in TSB-Salt were determined every hour by the pour-plate method using TSA-Salt with incubation at 35-37°C for 24 hr.

## 3.3 Oyster samples preparation

Raw Pacific oysters (average weight of 30.0±5.0 grams and shell length of 8.0±0.7 cm) obtained from Oregon Oyster Farms (Yaquina Bay, Newport, OR) were delivered to the laboratory in a cooler with ice on the day of harvest. Oysters were washed with tap water to remove mud on shell and then placed in a rectangular high-density polyethylene (HDPE) tank containing aerated artificial seawater (ASW) with a salinity of 30 ppt (parts per thousand). The ASW was prepared by dissolving Instant Ocean salt (Aquarium systems Inc., Mentor, OH) in deionized water according to manufacturer's instructions. Marine microalgae concentrate (Shellfish Diet 1800, Reed Mariculture Inc., Campbell, CA) was added to the ASW and oysters were held at room temperature overnight to help them regain biological activities before experiments.

### 3.4 Effects of pH value on oyster gaping

The ability of oysters to survive in seawater at various pH values were studied by holding six oysters in 15 L ASW of different pH values (4.0, 4.5, 5.0, 5.5; 9.0, 9.5, 10.0, 10.5) adjusted with acid (hydrochloride acid, lactic acid or citric acid; 5 mol/L) or sodium hydroxide (2.5 mol/L) at room temperature for 2 days. The mortality of oysters in ASW with different pH values were recorded daily. In addition, movement of oysters was monitored by a Gape-O-Meter (Pacific Shellfish Institute, Olympia, WA) at pH 5.5 and pH 9.0. The Gape-O-Meter consists of four rectangular bars each containing an electronic device capable of measuring the distance between the surface

of the bar and a magnetic sensor. For determination of oyster movement, six oysters were glued to the bars with a magnetic sensor attached to the upper shell of each oyster. The distance between the rectangular bar and magnetic sensor was recorded every 5 min for 48 hours. A change in the measurement of >0.03 inch (>0.05 cm) indicated a sign of oyster gaping.

### 3.5 Preparation of *Vibrio parahemolyticus* for oyster inoculation

*Vibrio parahemolyticus* strains were individually grown in 10 ml TSB-Salt and incubated overnight at 35-37°C. Each broth culture was streaked onto a plate of trypticase soy agar (TSA; Difco, Becton, Dickinson, Sparks, MD) containing 2% salt (TSA-Salt) and incubated at 35-37°C for 18-24 hr. One single colony formed on each TSA-Salt plate was transferred into 10 ml TSB-Salt and incubated at 35-37°C for 4 hr. Cells of *V. parahaemolyticus* in enriched TSB-Salt were poured into a 50-ml sterile centrifuge tube and harvested by centrifugation at 3,000×g (Sorvall RC-5B, Kendro Laboratory Products, Newton, CT) at 5°C for 15 min. Pellet cells were re-suspended in sterile 2% salt (NaCl) solution to obtain a *V. parahaemolyticus* culture cocktail of $10^{8\text{-}9}$ CFU/ml.

### 3.6 Inoculation of oyster with *Vibrio parahaemolyticus*

Oysters held in ASW at room temperature overnight were transferred to an identical tank of 20 L of fresh ASW (30 ppt) containing a mixture of five *V.*

*parahemolyticus* strains at a level of approximate $10^5$ CFU/ml. Accumulation of *V. parahaemolyticus* in oysters was conducted at room temperature overnight (16-18 h) to achieve a target level of contamination in oysters of about $10^{4-5}$ CFU/g. Air was also pumped into ASW to keep dissolved-oxygen (DO) levels favorable for oyster pumping and uptake of *V. parahaemolyticus*.

### 3.7 Impacts of temperature and pH value of ASW on depuration for decreasing *V. parahaemolyticus* in oysters

Oysters inoculated with *V. parahaemolyticus* were depurated for 5 days in a laboratory-scale re-circulating (1500 L/h) system equipped with a 15W Gamma UV sterilizer (Current-USA Inc., Vista, CA), a water chiller (Delta Star, Aqua Logic, Inc., San Diego, CA) and a temperature regulator at various temperature (12.5 or 20°C) and pH (5.5 or 7.0) combinations. A pH controller (BL6907, Hanna Instrument Inc., RI, US) was used to maintain the pH value of ASW at 5.5 or 7.0 by adding citric acid or lactic acid (0.5 mol/L) to ASW during the depuration process. Depuration of oysters in ASW (pH 8.3) without pH control was used as the control.

### 3.8 Impacts of oyster-water ratio on the efficacy of depuration in decreasing *V. parahaemolyticus* levels in oysters

To determine impacts of oyster-water ratio on the efficacy of depuration in decreasing *V. parahaemolyticus* levels in oysters, different volumes of ASW (40, 60,

80 and 100 L) were used for depurating 40 oysters inoculated with *V. parahaemolyticus* at 12.5°C for 5 days, resulting in various oyster-water ratios of one oyster per 1.0, 1.5, 2.0 or 2.5 L of ASW.

### 3.9 Analysis of *V. parahaemolyticus* in oysters during depuration

Densities of *V. parahemolyticus* in oysters during depuration were determined by the three-tube most-probable-number (MPN) method according to the Food and Drug Administration Bacteriological Analytical Manual (FDA, 2001). Five oysters were randomly picked from the depuration system every day and shucked with a sterile shucking knife. The oyster meat was homogenized with an equal volume of sterile alkaline phosphate buffer saline (PBS; pH 7.4) at high speed for 1 min using a two-speed laboratory blender (Waring Laboratory, Torrington, CT) to prepare 1:2 dilution sample suspensions. Twenty grams of the 1:2 dilution sample suspension were mixed with to 80 ml of PBS to make 1:10 sample dilution. Additional 10-fold dilutions were prepared with sterile PBS. All sample dilutions were individually inoculated into 3 tubes of alkaline peptone water (APW; pH 8.5; Difco, Becton Dickinson). Inoculated APW tubes were incubated at 35-37°C overnight. A 3-mm loopful from the top 1 cm of each turbid APW tube was streaked onto individual thiosulfate-citrate-bile salt-sucrose agar (TCBS; Difco, Becton Dickinson) plates and incubated at 35-37°C for 18-24 h. Formation of colonies that are round, green or bluish with 2-3 mm diameter on a TCBS plate after incubation was considered positive for *V. parahaemolyticus*.

Total populations of *V. parahemolyticus* in oysters were determined by converting the numbers of APW tubes that were positive for *V. parahemolyticus* to MPN per gram according to the 3-tube MPN table. The efficacy of the UV sterilizer in inactivating *V. parahaemolyticus* cells released from oysters into re-circulating ASW was verified daily by plating the ASW on TCBS plates followed by incubation at 37 ºC for 24 h.

### 3.10 Statistical analysis

Results of microbiological tests were converted to $\log_{10}$ values and analyzed with One-Way ANOVA and Tukey-Kramer multiple-comparison Test using the R program (R foundation, Vienna). Significant differences among means of each treatment over time were established at a level of $P<0.05$.

# Chapter 4. Results and Discussions

## 4.1 Effects of pH value on growth of *V. parahaemolyticus*

Changes of *V. parahaemolyticus* populations in TSB medium containing 2% salt with different pH values (5.5, 7.3 and 9.0) during incubation at 37°C are illustrated in Figure 4.1. All five pathogenic *V. parahaemolyticus* strains grew well at pH 7.3 and 9.0. The populations of *V. parahaemolyticus* increased by 5.9 to 6.8 log cfu/ml and 6.1 to 6.5 log cfu/ml after 8 h of incubation at 35-37°C at pH 7.3 and 9.0, respectively. No significant difference ($P$>0.05) was observed between the increases of *V. parahaemolyticus* populations grown in TSB-Salt at pH 7.3 and pH 9.0. However, smaller increases (1.7 to 5.2 log cfu/ml) in populations were observed when *V. parahaemolyticus* strains were grown in TSB-Salt of pH 5.5 at 37°C for 8 h. Growth of *V. parahaemolyticus* 10292, 10293 and O27-1C1 in TSB-Salt of pH 5.5 was slightly hindered with smaller increases in populations (4.72 cfu/ml for strain 10292, 5.22 cfu/ml for strain 10293, and 4.21 log cfu/ml for strain O27-1C1) being observed after 8 h of incubation when compared with those observed at pH 7.3 and 9.0. On the other hand, growth of strains 10290 and BE98-2029 in the same medium was significantly retarded with much smaller increases (1.73 log cfu/ml for 10290 and 2.40 log cfu/ml for strain BE98-2029) being observed. These results revealed that growth of certain strains of *V. parahaemolyticus* could be retarded at pH 5.5 and the

susceptibility of pathogenic strains of *V. parahaemolyticu* to low pH values varied among strains. Among the five pathogenic strains of *V. parahaemolyticus*, strain 10290 was the most sensitive to pH 5.5 followed by strain BE98-2029 while strain 10293 was the most resistant. The populations of strain 10290 increased by 6.1 and 6.5 log cfu/ml in TSB-Salt of pH 7.3 and 9.0, respectively, but only increased by 1.7 log cfu/ml in TSB-Salt of pH 5.5 after 8 h of incubation at 37°C.

*Vibrio parahaemolyticus* is known highly susceptible to low pH environment (FDA, 2012). It was reported that pH 4.8 was the lowest pH value for *V. parahaemolyticus* to grow (Beuchat, 1973). Even though a pH value of 5.5 could not inhibit the growth of this bacterium, it retarded the growth certain strains of pathogenic *V. parahaemolyticus*. Beuchat (1976) found that reducing the pH of TCBS by adding citric acid or malic acid to the medium resulted in extended lag phase and a decrease in total colonies formed by *V. parahaemolyticus* on TCBS plates. In shrimp homogenate, *V. parahaemolyticus* was shown to be very sensitive when the pH was decreased to below 6.0, but not at pH 6 to 10. (Vanderzant and Nickelson, 1973) A study of growth kinetics of four *V. parahaemolyticus* strains reported that all four strains grew much slower at pH 5.8 than at pH 7 or 8 at 20 or 25 °C (Nishina et al., 2004). In this study, we observed that growth of certain strains of pathogenic *V. parahaemolyticus* was significantly retarded in TSB-Salt of pH 5.5 at 35-37°C.

### 4.2 Effects of pH value on oyster gaping

To determine oyster's ability to survive when the pH values of the seawater changed, six Pacific oysters were held in ASW with a pH value ranging from 4.0 to 10.5 at room temperature for 2 days. The oysters survived well in ASW with pH values between 5.0 and 9.5 without a single death after 2 days (Table 4.1). However, all six oysters died within one day when the pH was reduced to 4.5 and below. Similarly, deaths of oysters were observed when they were held in ASW of pH 10.0 or higher.

The oyster gaping analyses were conducted in ASW of different pH values (5.5 and 9.0) to measure the magnitude of oyster movement in response to significant changes of the pH values of the growth environments. Oyster gaping in ASW of pH 5.5 adjusted with hydrochloride acid, citric acid and lactic acid are shown in Figure 4.2, 4.3 and 4.4, respectively. Oyster gaping was clearly observed at pH 5.5 regardless of the type of acid used for pH adjustment. Much more active oyster gaping was recorded when oysters were held in ASW with pH value adjusted with citric acid or lactic acid than in ASW adjusted with hydrochloric acid. However, the gaping of oysters appears to be largely dependent on individual oysters and varied a lot. Most of the oyster gaping recorded during the studies was between 0.2 to 0.5 inches. However, a maximum gaping of 0.8 inches was observed in one oyster held in ASW with pH value adjusted by lactic acid (Figure 4.4). It seems that oysters were less sensitive to a

pH change adjusted with citric acid or lactic acid. The gaping was actively recorded on the first day, but not the second day, for oysters held in ASW of pH 5.5 adjusted with hydrochloric acid (Figure 4.2). Oyster gaping in ASW at pH 9.0 is shown in Figure 4.5. Active oyster gaping was clearly observed for all six oysters with gaping ranging from 0.2-0.8 inches.

It is not clear whether oyster gaping is closely associated with the water pumping activity of oysters. However, this study demonstrated that the oysters could acclimate to a significant change of pH value in the growth environments and maintain biological activities.

Based on our findings that growth of *V. parahaemolyticus* was retarded at pH 5.5 and oyster gaping was actively recorded in ASW of pH 5.5, ASW of pH 5.5 adjusted with citric acid or lactic acid was selected to study impacts of temperature and pH value on depuration for decreasing *V. parahaemolyticus* levels in Pacific oysters.

### 4.3 Impact of temperature and pH value on depuration for reducing *V. parahaemolyticus* levels in Pacific oysters

Depuration studies for reducing *V. parahaemolyticus* contamination in oysters were conducted with ASW of two pH values (5.5 and 7.0) at two different temperatures (12.5 and 20°C). Most of the studies were conducted in wintertime, when the level of naturally accumulated *V. parahaemolyticus* in oysters was low. Depuration with ASW (pH 8.3) without pH control was used as the control.

The efficacy of depuration under various temperature (12.5 and 20°C) and pH value (pH 5.5, pH 7.0 and pH 8.3) conditions for reducing *V. parahaemolyticus* contamination in Pacific oysters was reported in Tables 4.2 and 4.3. Populations of *V. parahaemolyticus* in oysters were reduced by 2.82 log MPN/g after five days of depuration in ASW (pH 8.3) without pH control at room 20°C (Table 4.2). Smaller reductions of *V. parahaemolyticus* in oysters were observed after 5 days of depuration at 20°C with ASW of pH 5.5 (0.70-1.96 log MPN/g) or pH 7.0 (1.68-1.99 log MPN/g) with pH being controlled by addition of citric acid or lactic acid to ASW during the depuration process. In most of the processes, populations of *V. parahaemolyticus* decreased significantly after the first day of depuration and further decreased gradually thereafter. However, when conducting depuration at pH 5.5 controlled with citric acid, no significant reduction ($P>0.05$) of the *V. parahaemolyticus* populations in oysters was observed even after 5 days. These results indicate that reducing pH values of the ASW had a negative impact on the efficacy of depuration in decreasing *V. parahemolyticus* levels in Pacific oysters.

While significant reductions of *V. parahaemolyticus* populations in oysters were observed during depuration with ASW at 20°C without pH control, there was no difference observed for the reductions recorded after day 3 of the process. This may be due to the growth of *V. parahaemolyticus* in oyster tissues at 20°C. According to Parveen and others (2013), populations of *V. parahaemolyticus* in Eastern oyster

tissues decreased at 10°C, but could multiply in the oyster tissues at 15°C and 20°C with an average rate of 0.038 and 0.082 log cfu/g.

When the depuration temperature was decreased from 20°C to 12.5°C, greater reductions of *V. parahaemolyticus* in oysters were observed after 5 days of processes with ASW of pH 7.0 and pH 8.3 (control), but not pH 5.5 (Table 4.3). The reductions of *V. parahaemolyticus* in oysters by depuration at pH 5.5 were limited to 2.1 or 1.6 log MPN/g when the pH value of ASW was controlled by addition of citric acid or lactic acid. However, depuration at 12.5°C did increase reductions of *V. parahaemolyticus* levels in oysters with ASW of pH 7.0 and 8.3 with 2.9-3.0 and 3.5 log MPN/g reductions being observed after 5 days of depurations at pH 7.0 and 8.3, respectively (Table 4.3). These data confirm that reducing pH values of the ASW had a negative impact on the efficacy of depuration in decreasing *V. parahemolyticus* levels in Pacific oysters even when the processes were conducted with refrigerated ASW (12.5°C).

The process of depuration was holding oysters in clean seawater to allow oysters to purge contaminants from the digestive tract into water (Blogoslawski & Stewart, 1983). Therefore, the efficacy of depuration is highly dependent on oyster's physiological (water-pumping) activity. Oysters are known to adjust their biological activity in response to environmental changes (Loosanoff, 1958). Factors including water temperature (Loosanoff, 1958), water salinity (Rowse & Fleet, 1984), pH of

seawater (Loosanoff & Tommers, 1947), turbidity and dissolved oxygen level, had been investigated to affect the water pumping activity of oysters.

The Food Standards Agency of the United Kingdom recommended that Pacific oysters (*Crassostrea gigas*) be depurated at a temperature range between 8 and 18°C. (FAO, 2008) However, the United States National Shellfish Sanitation Program (NSSP) does not have a recommended temperature for oyster depuration. It is known that the populations of pathogens in shellfish are difficult to be reduced when shellfish is not actively pumping water (NSSP Guide for the Control of Molluscan Shellfish, 2011 revision). A study of water pumping of the Eastern oysters (*Crassostrea virginica*) observed a gradually increase in water pumping activity of oyster when water temperature rose from 8 to 16°C, and no further increase was observed between 16 and 28°C, but the activity dropped significantly at 2°C or lower (Loosanoff, 1958). Loosanoff (1958) reported that Gulf oysters could pump water at rates of 4-7 L/h at 15-20°C, but the rates gradually decreased to <1 L/h when water temperature decreased to 10°C. In this study, all depuration processes were conducted at 12.5 or 20°C and should have little or no impact on the water-pumping activity of oysters.

Many studies have reported that depuration at ambient temperatures had little effects on reducing *Vibrio* species in oysters. Tamplin and Cappers (1992) reported that depuration at 23°C did not reduce the *V. vulnificus* counts in artificially contaminated oysters, while an increase of the bacteria population was observed instead after 24 h. Another study found that depuration at 22°C reduced *V.*

*parahaemolyticus* by only 1.2 log MPN/g after 48 h of depuration (Chae et al., 2009). Phuvasate and others (2012) depurated artificially contaminated Pacific oysters in recirculating ASW at 2, 3, 7, 10, 12.5 and 15°C for 4-6 days and reported that >3.0 log MPN/g reductions could be achieved when oysters were depurated with UV-sterilized ASW (30 ppt) at 7 to 15°C for 5 days. Based on a reduction model developed in that study, the investigators predicted that depuration at 12.5°C for more than 5 days could achieve >3.52 log MPN/g reductions of *V. parahaemolyticus* in laboratory contaminated Pacific oysters. In this study, we hypothesized that reducing the pH value of ASW to 5.5 or 7.0 for depuration would improve the efficacy of the process for reducing *V. parahaemolyticus* in contaminated oysters. To investigate the hypothesis, two types of organic acids (citric acid and lactic acid) commonly used to adjust the pH values of food products for inhibiting bacteria growth (Huffman, 2002; Mahmound, 2014) were used to control the pH value of ASW during depuration studies. The mechanism of applying acids to inhibit microorganisms was to allow acids to penetrate the plasma membrane, thus acidifying the interior of the bacteria cells (Wang et al., 2013). Beuchat (1973) studied the sensitivity of *V. parahaemolyticus* to different acids, including citric acid, ascorbic and malic acid, and found that the degree of inhibition of *V. parahaemolyticus* growth depended on the pH of a medium, but not upon the acid added to the medium. In this study, we observed slightly larger reductions from depurations with ASW controlled by citric acid than lactic acid both at pH 5.5 (2.10 log MPN/g for citric acid and 1.63 log MPN/g for

citric acid) at pH 7.0 (3.01 log MPN/g for citric acid and 2.87 log MPN/g for citric acid) at 12.5°C than those observed at 20°C. However, such reductions were smaller than the reduction (3.52 log MPN/g) observed from depuration with ASW without pH control.

The pumping water activity of oysters may be affected by changes in pH values of the seawater. Loosanoff & Tommers (1947) found that oysters could pump normally at pH 7.75 and the pumping rate gradually decreased as the pH of water reduced to 6.5. At pH 4.14, the water pumping-rate of oysters was only 10% of the normal rate. This could explain why depuration processes were inefficient when the pH of ASW was reduced to 7.0 and 5.5. Even though our studies showed that growth of *V. parahaemolyticus* at pH 5.5 was retarded in TSB-Salt, the impact of pH value on *V. parahaemolyticus* growth was appears to be insignificant when compared with the negative impact of pH on the physiological activity of oysters on depuration for *V. parahaemolyticus* reduction in oysters. While oyster gaping was detected in ASW with pH value adjusted to 5.5, it's not clear whether oysters were actively pumping water under such a condition.

In summary, depuration with ASW of pH 5.5 or 7.0 was less efficient in reducing *V. parahaemolyticus* levels in oysters when compared with the process using ASW (pH 8.3) without pH adjustment. Nevertheless, we observed 3.52 log MPN/g reductions of *V. parahaemolyticus* in Pacific oysters after 5 days of depuration with ASW at 12.5°C (Table 4.3). This indicates that controlling the temperature but not the

pH value of water is critical to the efficacy of depuration for reducing *V. parahaemolyticus* in oysters.

### 4.4 Impacts of oyster-water ratio on the efficacy of depuration in decreasing *V. parahaemolyticus* levels in Pacific oysters

To investigate effects of water-oyster ratio on the efficacy of depuration for decontaminating *V. parahaemolyticus* in oysters, 40 oysters contaminated with *V. parahaemolyticus* were depurated at 12.5°C in 40, 60, 80 or 100 L of ASW, making an oyster-water (number of oyster / liter of ASW) ratio of 1:1, 1:1.5, 1:2 or 1:2.5. Reductions of *V. parahaemolyticus* in oysters after five days of processes are reported in Table 4.4. Among the different oyster-water ratios tested, the smallest reduction (2.45 log MPN/g) of *V. parahaemolyticus* in oysters waswas observed from depuration with 40 L of ASW. Increasing the water volume to 60 L for depuration resulted in a greater reduction of 3.54 (>3.52) log MPN/g of *V. parahaemolyticus* in oysters after 4 days of process. Further increase of water volume to 80 L produced 3.60 (>3.52) log MPN/g reduction of *V. parahaemolyticus* in oysters after 4 days of process and the reduction continued to increase to 3.91 log MPN/g after 5 days of process. When the water volume was increased to 100 L, a 2.99-log MPN/g reduction of *V. parahaemolyticus* in oysters was observed after 4 days of process. Based on these data, it was believed that depuration at 12.5°C with an oyster-water ratio of either 1:1.5 or 1:2 could deliver >3.52 reductions of *V. parahaemolyticus* in oysters after 4 days of

process. Two additional runs of depuration using 40 oysters with 60 or 80 L of ASW each were conducted to verify the efficacy of each depuration condition in decreasing *V. parahaemolyticus* in oysters. *V. parahaemolyticus* populations were reduced by 3.52 and 3.62 log MPN/g after 5 days of depuration with 60 L of ASW in both trials, but the reductions were less than 3.52 (3.27 and 3.45) log MPN/g after 4 days of processes (Table 4.5). However, populations of *V. parahaemolyticus* in oysters were decreased by >3.52 (3.55 and 3.93) log MPN/g after 4 days of depuration with 80 L of ASW in both trials with reductions being further increased to 3.92 and 4.11 log MPN/g after 5 days of processes. Further study of holding oysters in ASW at 12.5°C with an oyster-water ratio of 1:2 without removing oysters from the depuration tank until days 4 and 5 for analysis resulted in reductions of 3.75 and 4.00 log MPN/g of *V. parahaemolyticus* in Pacific oysters, respectively. This demonstrates that depuration at 12.5°C with an oyster-water ratio of 1:2 can achieve greater than 3.52 log MPN/g reductions of *V. parahaemolyticus* in oysters in 4 days, which may be applied by the shellfish industry as a post-harvest process of shellfish to achieve >3.52 log MPN/g reductions of *V. parahaemolyticus* in oysters established by the National Shellfish Sanitation Program (FDA 2009).

A processor using depuration treatments to reduce the *V. parahaemolyticus* risk to consumers of raw oysters would need to comply with U.S. FDA requirements specifying “that the process reduces the level of *Vibrio parahaemolyticus* to non-detectable (<30 MPN/gram) and that the process achieves a minimum 3.52 log

reduction (NSSP, 2013).” Furthermore, *V. parahaemolyticus* levels would need to be determined following the sampling protocol (NSSP, 2013) and microbial enumeration (NSSP, 2013) described by the National Shellfish Sanitation Program (NSSP) which was followed in this study. The 3.52 log reduction in these regulations is based on assuming *V. parahaemolyticus* loads observed sometimes in the Gulf Coast during summer months (100,000 MPN/g) being lowered by depuration or other alternatives to reach non-detectable levels (<30 MPN/g) (NSSP, 2013). Although in depuration tests at the optimum pH and oyster numbers to seawater ratio determined in this study, SV levels estimated as the difference in the average load of untreated oysters and that after 5 day depuration time exceeded the required 3.52 log reductions, the statistical variability of these SV levels was not determined. The statistical assessment of reducing the *V. vulnificus* risk when consuming raw oysters by depuration (Deng et al., 2015) and high pressure processing (Serment-Moreno et al., 2015) treatments was conducted following a Monte Carlo protocol. A similar assessment might be used to further analyze the depuration data obtained in this study, and including that reported in Appendix A, to determine the statistical variability of the SV levels achieved in the *V. parahaemolyticus* load in raw oysters when optimizing the depuration pH and oyster numbers to seawater ratio. Nevertheless, the depuration process for reducing *V. parahaemolyticus* in Pacific oysters by >3.52 log units needs to be validated according to the National Shellfish Sanitation Program’s postharvest processing validation–verification interim guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus* for

application by the shellfish industry as a post-harvest processing to reduce *V. parahaemolyticus* in raw oysters.

In summary, decreasing pH value of seawater for depuration resulted in decreased efficacy of depuration process for reducing *V. parahaemolyticus* contamination in oysters. Oyster-water ratio plays a role in enhencing the efficacy of depuration for decreasing *V. parahaemolyticus* levels in oysters. In this study, we identified a oyster-water ratio capable of delivering >3.52 log MPN/g reductions of *V. parahaemolyticus* in Pacific oysters after 4 days of depuration at 12.5°C. This new discovery has a great potential to be applied by shellfish industry as a post-harvest processing to produce safe oysters for raw consumption.

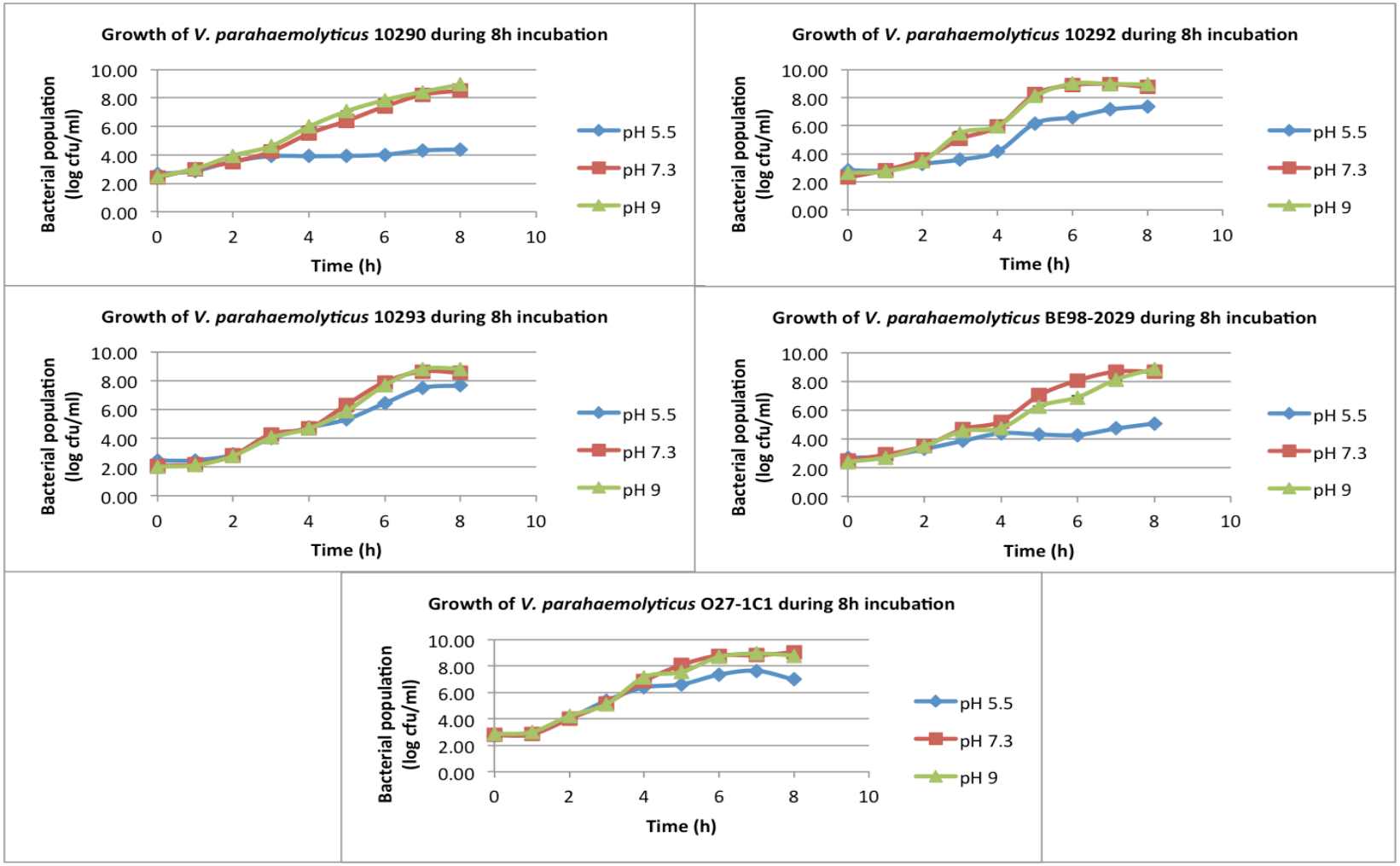


Figure 4.1 Growth of five pathogenic *V. parahaemolyticus* strains in TSB-salt medium at different pH values. (The error bars on each data was added but too small to be seen)

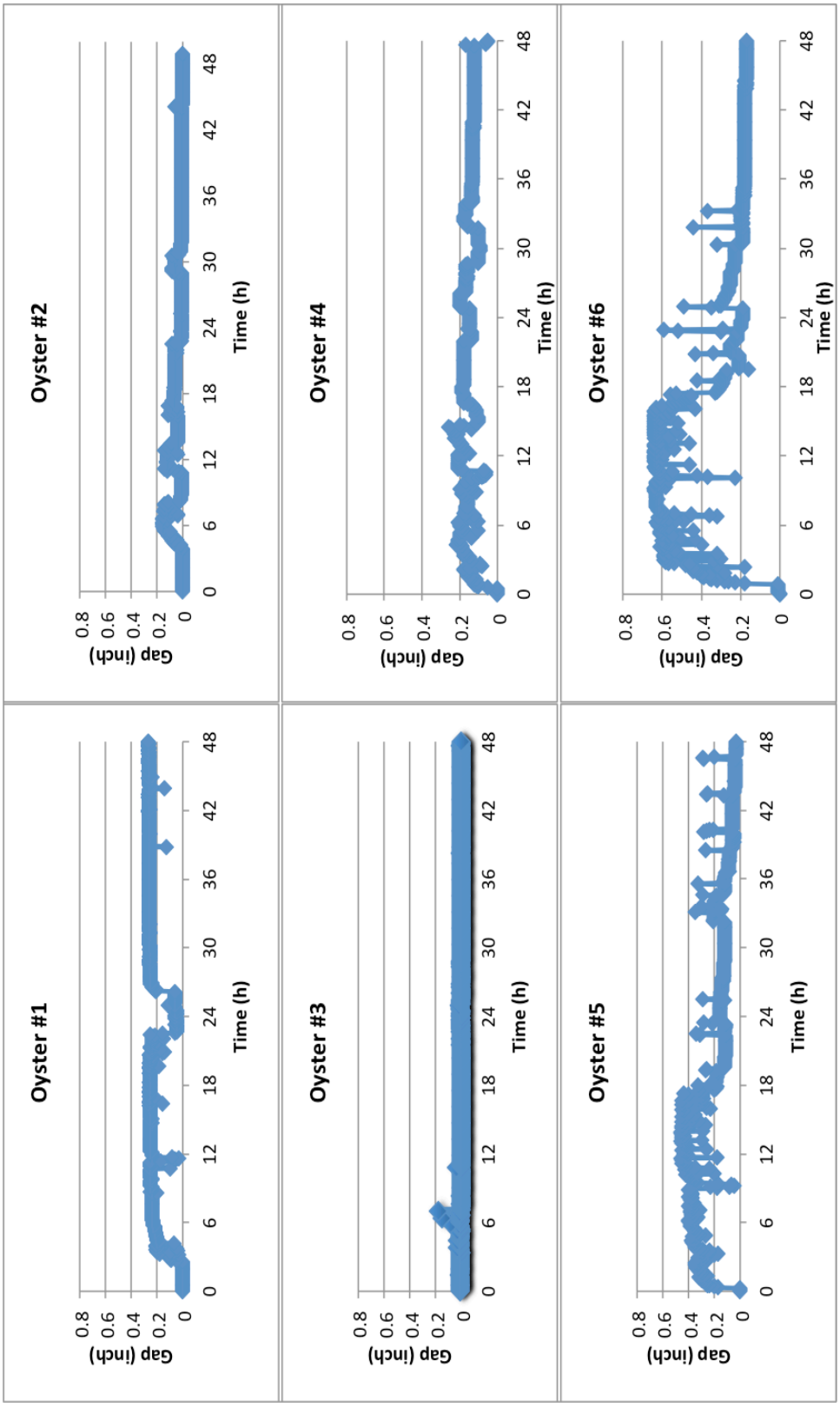


Figure 4.2. Oyster gaping at room temperature in ASW (30 ppt) of pH 5.5 adjusted with hydrochloride acid.

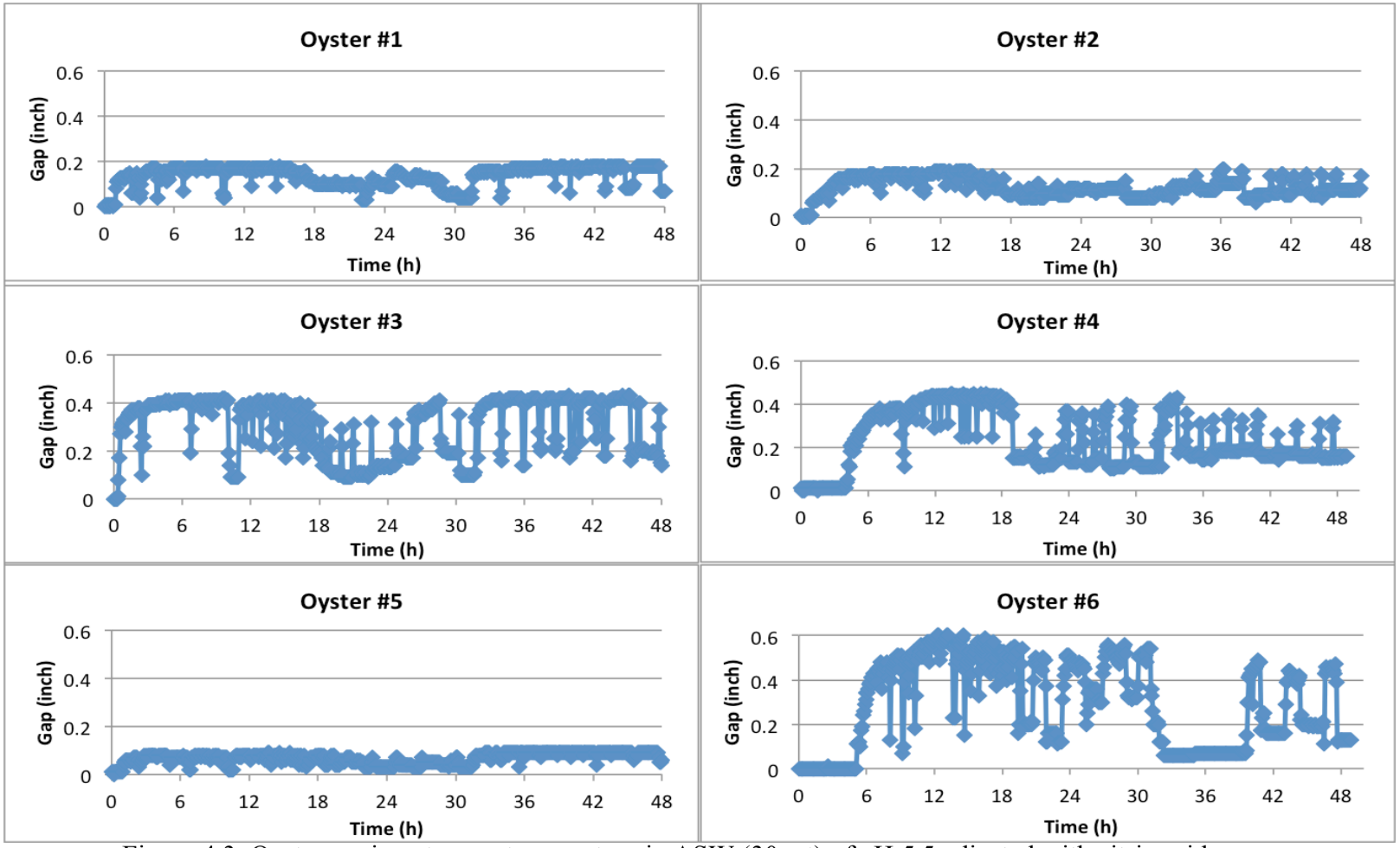

Figure 4.3. Oyster gaping at room temperature in ASW (30ppt) of pH 5.5 adjusted with citric acid

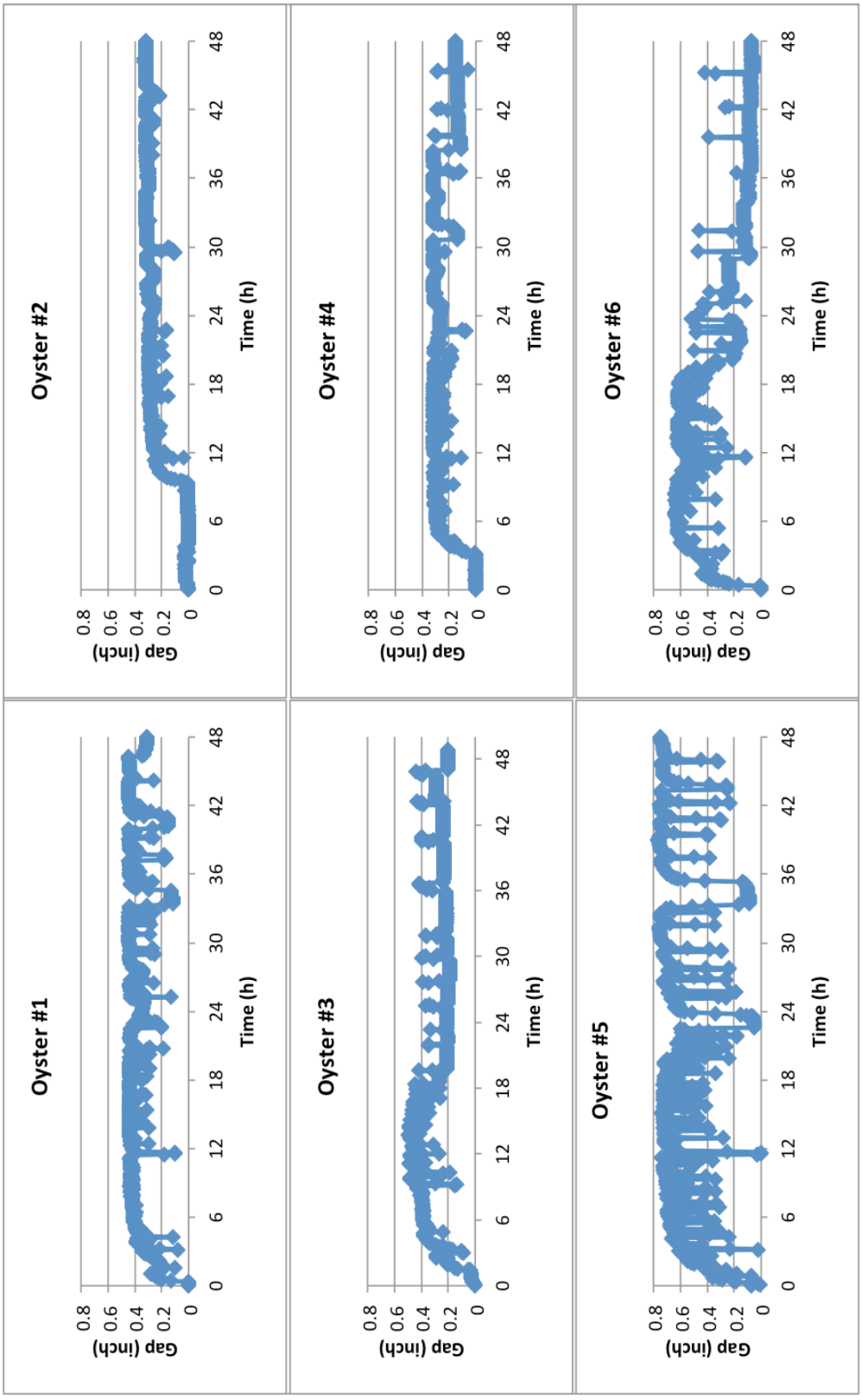


Figure 4.4. Oyster gaping at room temperature in ASW (30ppt) of pH 5.5 adjusted with lactic acid

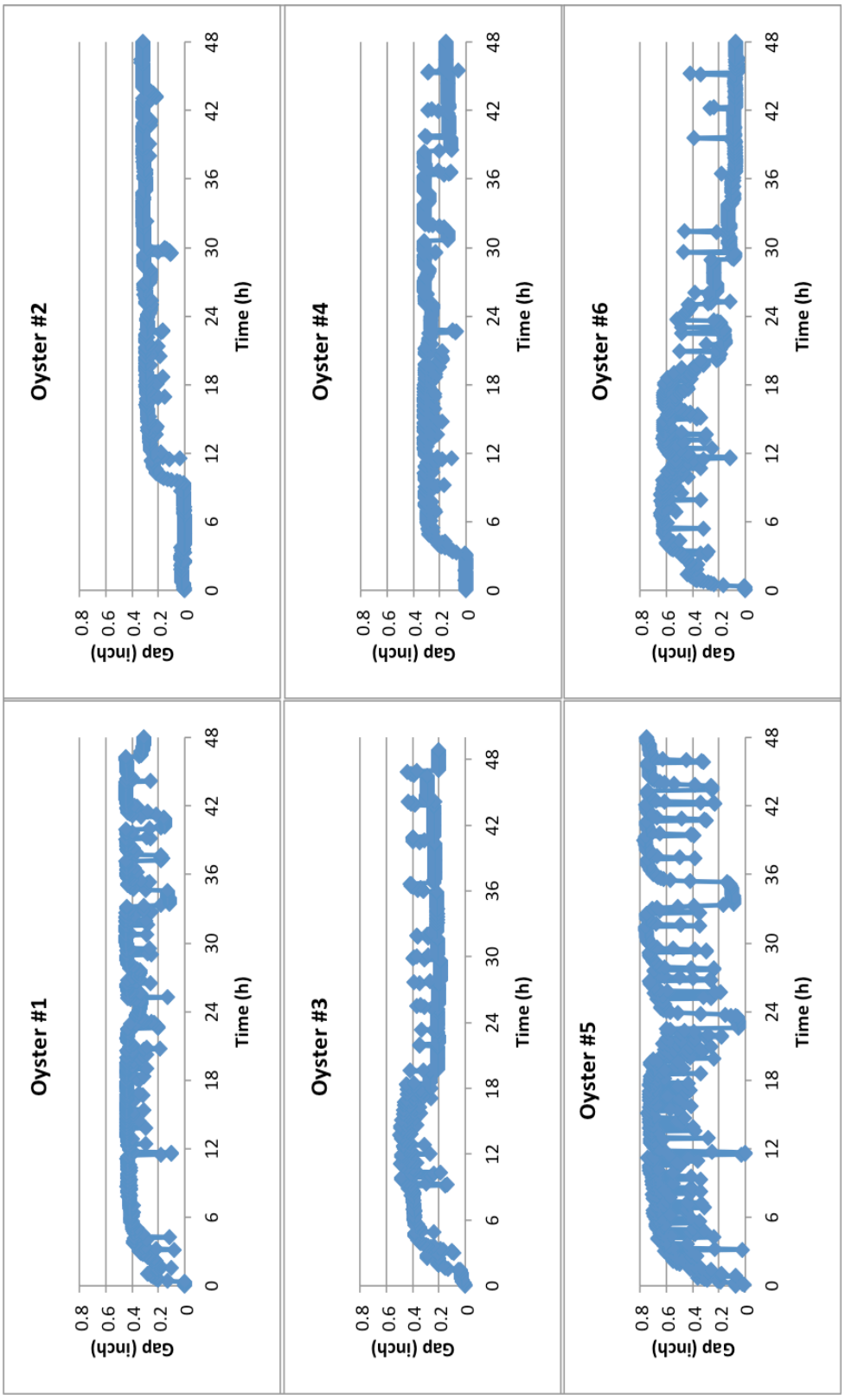


Figure 4.5 Oyster gaping at room temperature in ASW (30ppt) of pH 9.0 adjusted with sodium hydroxide

Table 4.1 Survival of oysters in ASW of different pH values at room temperature for 2 days.

| pH* | Oyster Mortality** |
|---|---|
| 4.0 | 6/6 |
| 4.5 | 6/6 |
| 5.0 | 0/6 |
| 5.5 | 0/6 |
| 9.0 | 0/6 |
| 9.5 | 0/6 |
| 10.0 | 2/6 |
| 10.5 | 6/6 |

* Hydrochloride acid was used to adjust the pH to 4.0-5.5. Sodium hydroxide was used to adjust the pH to 9.0-10.5.
** Number of oysters died/ number of total oysters tested.

Table 4.2. Changes of *V. parahaemolyticus* population during depuration with ASW of different pH values at 20°C.

| Days | pH 5.5 | | pH 8.3 | pH 7.0 | |
|---|---|---|---|---|---|
| | Trial 1[a] | Trial 2[b] | | Trial 1[a] | Trial 2[b] |
| 0 | 5.36±0.18A[c] | 5.80±0.28A | 5.43±0.39A | 5.23±0.47A | 5.06±0.19A |
| 1 | 5.35±0.44A(0.01[d]) | 4.34±0.50B(1.46) | 3.49±0.65B(1.94) | 4.38±0.36B(0.85) | 4.43±0.38B(0.63) |
| 2 | 4.78±0.37A(0.58) | 4.21±0.71B(1.60) | 3.44±0.56B(1.99) | 4.19±0.40C(1.04) | 3.71±0.48C(1.34) |
| 3 | 5.10±0.46A(0.26) | 4.07±0.64B(1.73) | 3.01±0.51B(2.42) | 4.06±0.46C(1.17) | 3.31±0.38CD(1.74) |
| 4 | 5.26±0.53A(0.10) | 4.18±0.55B(1.62) | 2.81±0.32B(2.62) | 4.05±0.46C(1.17) | 3.10±0.47CD(1.95) |
| 5 | 4.66±0.50A(0.70) | 3.85±0.47B(1.96) | 2.61±0.45B(2.82) | 3.55±0.60C(1.68) | 3.07±0.10D(1.99) |

[a] Trial 1: pH 5.5 of ASW adjusted with citric acid.
[b] Trial 2: pH 5.5 of ASW adjusted with lactic acid.
[c] Data are means of five determination ± standard deviation. Means with different letters in the same column are significantly different ($p<0.05$).
[d] Reductions in bacterial population (log MPN/g).

Table 4.3. Changes of *V. parahaemolyticus* population during depuration with ASW of different pH values at 12.5°C.

| Days | pH 5.5 | | pH 8.3 | pH 7.0 | |
|---|---|---|---|---|---|
| | Trial 1[a] | Trial 2[a] | | Trial 1[a] | Trial 2[b] |
| 0 | 5.80±0.28A[b] | 5.41±0.36A | 5.28±0.27A | 5.07±0.37A | 5.39±0.32A |
| 1 | 4.51±0.64B (1.30[c]) | 4.76±0.46B(0.65) | 3.23±0.49B (2.05) | 3.77±0.18B(1.30) | 4.99±0.62AB(0.40) |
| 2 | 4.24±0.52B (1.56) | 4.62±0.12B(0.80) | 2.90±0.42B (2.39) | 3.02±0.44B(2.05) | 3.96±0.50B(1.43) |
| 3 | 3.94±0.63B (1.87) | 4.02±0.21C(1.39) | 2.67±0.48BC (2.61) | 2.68±0.34C(2.38) | 2.98±0.45C(2.41) |
| 4 | 3.79±0.43B (2.01) | 3.79±0.17C(1.63) | 2.01±0.19CD (3.27) | 2.36±0.34CD(2.71) | 2.64±0.45C(2.74) |
| 5 | 3.70±0.56B (2.10) | 3.78±0.51C(1.63) | 1.76±0.76D (3.52) | 2.06±0.37D(3.01) | 2.52±0.16C(2.87) |

[a] Trial 1 indicated oyster depuration at pH 5.5 in ASW adjusted with Citric Acid, Trial 2 indicated oyster depurations at pH 5.5 in ASW adjusted with Lactic Acid.
[b] Data are means of five determination ± standard deviation. Means with different letters in the same column are significantly different ($p<0.05$).
[c] Reductions in bacterial population (log MPN/g).

sTable 4.4. Changes of *V.parahaemolyticus* population during depuration using different oyster-water ratios at 12.5°C.

| Days | Depuration with 40L ASW (1:1) (log MPN/g) | Depuration with 60L ASW (1:1.5) (log MPN/g) | Depuration with 80L ASW (1:2) (log MPN/g) | Depuration with 100L ASW (1:2.5) (log MPN/g) |
|---|---|---|---|---|
| 0 | 5.00±0.45A[a] | 4.83±0.18A | 5.27±0.47A | 4.97±0.42A |
| 1 | 4.07±0.32B (0.94[b]) | 3.12±0.39B (1.71) | 3.10±0.41B (1.97) | 2.97±0.27B (2.01) |
| 2 | 3.78±0.36BC (1.23) | 1.77±0.36C (3.07) | 2.45±0.55BC (2.62) | 2.78±0.38C (2.19) |
| 3 | 3.22±0.23CD (1.79) | 1.68±0.22CD (3.15) | 2.15±0.34CD (2.92) | 2.61±0.13CD (2.37) |
| 4 | 2.88±0.24DE(2.13) | 1.29±0.32CD (3.54) | 1.47±0.15DE (3.60) | 1.98±0.38CD (2.99) |
| 5 | 2.55±0.15E (2.45) | 1.09±0.37D (3.74) | 1.16±0.45E (3.91) | 1.94±0.39D (3.03) |

[a] Data are means of five determination ± standard deviation. Means with different letters in the same column are significantly different ($p<0.05$).

[b] Reductions in bacterial population (log MPN/g).

Table 4.5. *V. parahaemolyticus* population changes during two trials of depuration at different oyster-water ratio (1:1.5, 1:2)

| Days | Depuration with 60L ASW (1:1.5) (log MPN/g) | | Depuration with 80L ASW (1:2) (log MPN/g) | |
|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| 0 | 5.28±0.27A[a] | 5.16±0.39A | 5.60±0.41A[a] | 5.07±0.32A |
| 1 | 3.23±0.49B(2.05[b]) | 2.63±0.33B(2.53) | 3.12±0.40B(2.48[b]) | 2.47±0.15B(2.60) |
| 2 | 2.90±0.42B(2.39) | 2.58±0.25B(2.58) | 2.92±0.27B(2.69) | 1.31±0.53C(3.76) |
| 3 | 2.67±0.48BC(2.61) | 2.20±0.66BC(3.06) | 2.45±0.45B(3.15) | 1.34±0.40C(3.73) |
| 4 | 2.01±0.19CD(3.27) | 1.71±0.26C(3.45) | 2.05±0.22C(3.55) | 1.14±0.23C(3.93) |
| 5 | 1.76±0.76D(3.52) | 1.54±0.43C(3.62) | 1.69±0.32C(3.92) | 0.96±0.40C(4.11) |

[a] Data are means of five determination ± standard deviation. Means with different letters in the same column are significantly different ($p<0.05$).

[b] Reductions in bacterial population (log MPN/g).

# Chapter 5. Conclusion

*Vibrio parahaemolyticus* is the leading cause of human gastroenteritis associated with seafood, particularly raw oyster, consumption. The aim of this study was to investigate effects of factors, such as pH value of water and oyster-water ratio, on depuration process in decreasing *V. parahaemolyticus* levels in Pacific oysters and indentify an optimal depuration condition as a post-harvest treatment to reduce *V. parahaemolyticus* in oysters, which will decrease risks of *V. parahaemolyticus* associated with raw oyster consumption.

Initial study of five clinical *V. parahaemolyticus* strains [(10290 (O4:K12), 10292 (O6:K18), 10293 (O1:K56), BE98-2029 (O3:K6)] for their ability to grow in Trypticase Soy Broth with 2% salt (TSB-Salt) medium of various pH values (5.5, 7.3 and 9.0) revealed that all *V. parahaemolyticus* strains grew well at pH 7.3 and pH 9.0, but growth of all five strains was retarded in TSB-Salt of pH 5.5 when incubated at 37°C. Further studies of the ability of the Pacific oysters to survive in artificial seawater (ASW) of different pH values ranging from 4.0 to 10.5 unveiled that the oysters survived well in ASW of pH between 5.0 and 9.5 at room temperature for 2 days. Based on these results, depurations of oysters with ASW of pH 5.5 and 7.0 were investigated for the efficacy in decreasing *V. parahaemolyticus* in contaminated Pacific oysters at 12.5 and 20°C. Results were compared with those observed from

depuration with ASW (pH 8.3) without pH adjustment. The efficacy of depuration in decreasing *V. parahaemolyticus* in contaminated Pacific oysters was significantly enhanced by lowering water temperature from 20 to 12.5°C from in all studies. Depuration at 12.5°C with ASW without pH adjustment for five days delivered 3.52 log MPN/g reductions of *V. parahaemolyticus* in oysters. Lowering the pH of ASW to 5.5 or 7.0 for depuration at the same condition resulted in smaller reductions (1.63-2.10 log MPN/g at pH 5.5 and 2.87-3.01 log MPN/g at pH 9.0), indicating a negative impact of ASW with reduced pH values on the efficacy in decreasing *V. parahaemolyticus* in oysters.

Studies of the impact of oyster to water (ASW) ratio on the efficacy of depuration in decreasing *V. parahaemolyticus* levels in oysters identified an oyster to water ratio of 1:2 (number of oyster : liter of water) improved the efficacy of depuration that achieved >3.52 log MPN/g reductions after four days of depuration at 12.5°C. This new discovery improves the efficacy of depuration with ASW for reducing *V. parahaemolyticus* in Pacific oysters with >3.52 log MPN/g reductions of *V. parahaemolyticus* in oysters being achieved after four days of processing.

In summary, controlling the water temperature and oyster to water ratio plays an important role in the efficacy of depuration in reducing *V. parahaemolyticus* levels in oysters. Depuration with ASW at 12.5°C with oyster to water ratio of 1:2 may be developed as a post-harvest process to decrease *V. parahaemolyticus* contamination in

raw oysters by >3.52 log MPN/g. Future studies are needed to validate the efficacy of this controlled depuration for commercial application by the shellfish industry.

# Bibliography

Altekruse, S.F., Bishop, R.D., Baldy, L.M., Thompson, S.G., Wilson, S.A., Ray, B.J., Griffin, P.M. (2000). *Vibrio* gastroenteritis in the US Gulf of Mexico region: the role of raw oysters. Epidemiol. Infect., 124, 489-495.

Andrews, L., Jahncke, M., Mallikarjunan, K. (2003). Low dose Gamma irradiation to reduce pathogenic *Vibrio*s in live oysters (*Crassostrea virginica*). Journal of Aquatic Food Product Technology, 12, 71-82.

Andrews, L., Posadas, B. (2003). Oyster irradiation: pathogenic *Vibrio* response and consumer difference testing. Sea Grant Report #MASGP-02-016, Feb 13, 2003.

Andrews, L.S., Park, D.L., Chen, Y.P. (2000). Low temperature pasteurization to reduce the risk of *Vibrio* infection from raw shell-stock oysters. Food Additives and Contaminants, 19, 787-791.

Berlin, D.L., Herson, D.S., Hicks, D.T., Hoover, D.G. (1999). Response of pathogenic *Vibrio* species to high hydrostatic pressure. Applied and environmental microbiology. 65: 2776-2780.

Beuchat, L.R. (1973). Interacting Effects of pH, temperature, and salt concentration on growth and survival of *Vibrio parahaemolyticus*. Applied Microbiolog, 25, 844-846.

Beuchat, L.R. (1976). Sensitivity of *Vibrio parahaemolyticus* to spices and organic acids. Journal of Food Sience. 41, 899-902.

Blake, P. (1983). Vibrios on the half shell: what the walrus and carpenter didn't know. Ann Intern Med., 99, 558-559.

Blogoslawski, W.J., Stewart, M.E. (1983). Depuration and public health. J. World Maric. Soc., 14, 353-545.

Bubb, H.D. (1975). *Vibrio parahaemolyticus*—a marine pathogen detected in South African coastal waters. S. Afr. Med. J., 49, 1514-1516

Burkhardt, W., Calci, K.R. (2000). Selective accumulation may account for shellfish-associated viral illness. Applied and Environmental microbiology, 66, 1375-1378.

Caburlotto, G., Chidini, V., Gennari, M., Tafi, M.C., Lleo, M.M. (2008). Isolation of a *Vibrio parahaemolyticus* pandemic strain from a marine water sample obtained in the northern Adriatic. Eurosurveillance, 11, 1-2.

Caburlotto, G., Haley, B.J., Lleo, M.M., Huq, A., Colwell, R.R. (2010). Serodiversity and ecological distribution of *Vibrio parahaemolyticus* in the Venetian Lagoon, Northeast Italy. Environmental microbiology reports, 2, 151-157.

[CDC] Center for Disease Control and Prevention (CDC). (2014). Estimates of foodborne illnesses in the United States. Available at: http://www.cdc.gov/foodborneburden/index.htm

[CDC] Center for Disease Control and Prevention. (2012). Pandemic *Vibrio parahaemolyticus*, Maryland, USA, Emerging Infectious Disease Journal. 20. Available at: http://www.cdc.gov/media/eid/2014/4.html

[CDC] Center for Disease Control and Prevention. (2013). Increase in *Vibrio parahaemolyticus* illnesses associated with consumption of shellfish from several Atlantic coast harvest areas, United States. Available from: www.cdc.gov/*Vibrio*/investigations/.

[CDC] Center for Diseases Control and Prevention. (1998). Outbreak of *Vibrio parahaemolyticus* infection with eating raw oysters—Pacific Northwest, 1997. Morbid Mortal Weekly Rep, 47, 457-462.

[CDC] Centers for Disease Control and Prevention (CDC). (2013). Gould L.H., Walsh K.A., Vieira A.R., Herman K., Williams I.T., Hall A.J., Cole D. Surveillance for Foodborne Disease Outbreaks-United States, 1998-2008. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/ss6202a1.htm

[CDC] Centers for Disease Control and Prevention. (2013). Overview. Available at: http://www.cdc.gov/norovirus/about/overview.html

[CDC] Centers for Disease Controls and Prevention (CDC). (2011). National Enteric Disease Surveillance: COVIS Annual Summary, 2011. Available at: http://www.cdc.gov/ncezid/dfwed/PDFs/covis-annual-report-2011-508c.pdf

[CDC] Centers for Diseases Control and Prevention. (1999). Outbreak of *Vibrio parahaemolyticus* infection with eating raw oysters and clams harvested from Long Island Soung-Connecticut, New Jersey and New York, 1998. Morbid Mortal Wkly Rep, 48, 48-51.

[CDC] Centers for Diseases Control and Prevention. (2012). Vibrio illnesses (Vibriosis). Available at : http://www.cdc.gov/*Vibrio*/*Vibrio*p.html

Chae, M.J., Cheney, D., Su, Y.-C.. (2009). Temperature Effects on the depuration of *Vibrio parahaemolyticus* and *Vibrio vulnificus* from the American oyster (*Crassostrea virginica*). Food Microbiology and Safety (74): 62-66.

Chen, C.Y., Evers, D.C., Fleishman, B.J., Mason, R.P., Serrell, N.A., Lambert, K.F., Bank, M.S. (2008). Meeting report: methylmercury in marine ecosystems-from sources to seafood consumers. Environmental Health Perspectives. 116(12): 1706-1712.

Chen, C.Y., Serrell, N., Evers, D.C., Fleishman, B.J., Lambert, K.F., Weiss, J., Mason, R.P., Bank, M.S., (2008). Meeting Report: Methylmercury in marine ecosystems – from sources to seafood consumers. Environmental Health Perspectives. 116, p1706-1712.

Chen, Y.P. (1996). Reduction and management of *Vibrio vulnificus* in Gulf coast Oyster Orassostrea virginica. PhD Dissertation, Louisiana State University. 94.

Croci, L., Suffredini, E., Cozzi, L., Toti, L. (2001). Effects of depuration of molluscs experimentally contaminated with *Escherichia coli*, *Vibrio cholerae* O1 and *Vibrio parahaemolyticus*. Journal of Applied Microbiology, 92, 460-465.

Daniels, N.A., MacKinnon, L., Bishop, R., Altekruse S., Ray, B., Hammond, R.M., Thompson, S., Wilsom, S., Bean, N.H., Griffin, P.M., Slutsken, L. (2000). *Vibrio parahaemolyticus* Infections in the United States, 1973–1998. The Journal of Infectious Diseases. 181: 1661-1666.

Daniels, N.A., Ray, B., Eason, A., Marano, N., Kahn, E., McShan, A.L., Rosario, L.D., Baldwin, T., Kingsley, M.A., Puhr, N.D., Wells, J.G., Augulo, F.J. (2000). Emergence of a new O3:K6 *Vibrio parahaemolyticus* serotype in raw oysters. JAMA, 284, 1541-1545.

Deng K., Wu X., Fuentes C., Su Y.-C., Welti-Chanes J., Paredes-Sabja D., Torres J.A. 2015. Risk analysis of depuration treatments during the *Vibrio vulnificus* load in raw oysters. Journal of Food Protection, 78, 1113-1118.

DePaola A., Nordstrom J.L., Bowers J.C., Wells J.G., Cook D.W. (2003). Seasonal Abundance of Total and Pathogenic *Vibrio parahaemolyticus* in Alabama Oysters. Applied and Environmental Microbiology, 69, 1521-1526.

DePaola A., Ulaszek J., Kaysner C.A., Tenge B.J., Nordstrom J.L., Wells J., Puhr N., Gendel S.M. (2003). Molecular, serological, and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food, and clinical sources in North America and Asia. Applied and Environmental Microbiology, 69, 3999-4005.

DePaola, A., Kaysner, C.A., Bower, J., Cook, D.W. (2000). Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas and New York. (1997 and 1998). Appl. Environ. Microbiol. 2000, 66(11):4649-4654.

DePaulo A., Hopkins L.H., Peeler J.T., Wentz B., McPhearson R.M. (1990). Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. Applied and Environmental Microbiology. 56: 2299-2302.

Domingo J.L., Bocio A., Falco G., Llobet J.M. (2007). Benefits and risks of fish consumption Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminatants. Toxicology, 230, 219-226.

Drake, S.L., DePaola, A., Jaykus, L. (2007). An Overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Comprehensive Review in Food Science and Food Safety, 6, 120-144.

Duan, J., Su, Y. (2005). Occurrence of *Vibrio parahaemolyticus* in Two Oregon Oyster-growing Bays. Journal of Food Science, 70, 58-63.

European Commission. (2006). Commission regulation (EC) no. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff. Official Journal of the European Union L364/5. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF.

Eyles, M.J., Davey, G.R. (1984). Microbiology of Commercial Depuration of the Sydney Rock Oyster, *Crassostrea commercialis*. J Food Prot, 47, 703-706.

[FAO/WHO] Food and Agriculture Organization of the United Nations/ World Health Organization. (2011). Risk assessment of *Vibrio parahaemolyticus* in seafood: Interpretative summary and Technical report. Microbiological Risk Assessment Series. No. 16. Rome. 193pp.

[FAO] Food and Agriculture Organization of the United Nations, (2014). Available at: http://www.fao.org/fishery/statistics/global-consumption/en

[FDA.] Food and Drug Administration. (2005). Interpretative summary: Quantitative risk assessment on the public health impact of pathogenic *Vibrio parahaemolyticus* in raw oysters.

[FDA.] Food and Drug Administration. (2004). Mercury levels in commercial fish and shellfish. Available at: http://www.fda.gov/Food/FoodborneIllnessContaminants/Metals/ucm115644.htm

[FDA.] Food and Drug Administration. (2004). What you need to know about mercury in fish and shellfish. Available at: http://www.fda.gov/Food/FoodborneIllnessContaminants/Metals/ucm351781.htm

[FDA.] Food and Drug Administration. (2011). Fish and fisheries products harzards and controls guidance. Center for Food Safety & Applied Nutrition.

[FDA.] Food and Drug Administration. (2012). Bad Bug Book: Foodborne Pathogenic Microorganisms and Natural Toxins (2 ed.). Available at: http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/ucm2006773.htm

[FDA] Food and Drug Administration. (2004). What you need to know about mercury in fish and shellfish. Available at: http://www.fda.gov/Food/FoodborneIllnessContaminants/Metals/ucm351781.htm

[FDA] Food and Drug Administration. (2014). Irradiation in the Production, Processing and Handling of Food. https://www.federalregister.gov/articles/2014/04/14/2014-07926/irradiation-in-theproduction-processing-and-handling-of-food.

Feely, R.A., Alin, S.R., Newton, J., Sabine, C.L., Warner, M., Devol, A., Krembs, C., Maloy, C. 2010. The combined effects of ocean acidification, mixing, and respiration on pH and carbonate saturation in an urbanized estuary. Estuarine, Coastal and Shelf Science, 88, 442-449.

Food Consumption Reference: U.S. Census Bureau, (2011). Statistical Abstract, Health & Nutrition: Food Consumption and Nutrition.

(http://www.census.gov/compendia/statab/cats/health_nutrition/food_consumption_and_nutrition.html)

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

Garthwaite, I. (2000). Keeping shellfish safe to eat: a brief review of shellfish toxins, and methods for their detection. Trends in Food Science & Technology, 11, 235-244.

Gessner, B.D., Bell, P., Doucette, G.I., Moczydlowski, E., Poli, M.A., Dolah, F.V., Hall, S. (1997). Hypertension and identification of toxin in human urine and serum following a cluster of mussel-associated paralytic shellfish poisoning outbreaks. Toxicon, 35, 711-722.

Gooch, J.A., DePaola, A., Bower, J., Marshall, D.L. (2002). Growth and Survival of *Vibrio parahaemolyticus* in Postharvest American Oysters. Journal of Food Protection, 65, 970-974.

Han, B., Jeng, W.L., Chen, R.Y., Fang, G.T., Hung, T.C., Tseng, R.J. (1998). Estimation of target harzard quotients and potential health risks for metals by consumption of seafood in Taiwan. Arch. Environ. Contam. Toxicol., 35, 711-720.

Hara-Kudo, Y., Sugiyama, K., Nishibuchi, M., Chowdhury, A., Yatsuyanagi, J., Ohtomo, Y., Saito, A., Nagano, H., Nishina, T., Nakagawa, H., Konuma, H., Miyahara, M., Kumagai, S. (2003). Prevalence of pandemic thermostable direct hemolysin-producing *Vibrio parahaemolyticus* O3:K6 in seafood and the coastal environment in Japan. Applied And Environmental Microbiology. 69, 3883-3891.

Hardesty, S. (2001). Marketing opportunities for Pacific coast oysters. Pacific coast shellfish growers association, Food Marketing and Economics Group, Davis, California.

He, H., Adams, R.M., Farkas, D.F., Morrisey, M.T. (2000). Use of high-pressure processing for oyster shucking and shelf-life extension. Journal of Food Science, 67, 640-645.

Hellberg, R.S., DeWitt, C.A.M., Morrissey, M.T. (2012) Risk-benefit analysis of seafood consumption: A review. Comprehensive reviews for food science and food safety., 11, 490-517.

Hlady, W.G., Klontz, K.C. (1996). The Epidemiology of *Vibrio* Infections in Florida,

1981-1993. J. of Infection Disease., 173, 1176-1183.

Honda, T., Ni, X., Miwatani, T. (1988). Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infection and Immunity, 56,961-965.

Hood, M.A., Ness, G.E., Rodrick, G.E., Blake, N.J. (1983). Effects of storage on microbial loads of two commercially important shellfish species, *Crassostrea virginica* and *Mercenaria campechiensis*. Applied and Environmental Microbiology, 45, 1221-1228.

Huffman, R.D. (2002). Current and future technologies for the decontamination of carcass and fresh meat. Meat Science, 62, 285-294.

[IDSC] Infectious Disease Surveillance Center (1999). *Vibrio parahaemolyticus*, Japan, 1996-1998. Infectious Agents Surveillance Report, Vol. 20, No. 7, Ministry of Health, Labour, and Welfare, Japan. Available at: http://idsc.nih.go.jp/iasr/20/233/tpc233.html

[ISSC] Interstate Shellfish Sanitation Conference (1997). National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish. Washington, DC.

Iwamoto, M., Ayers T., Mahon, B.E., Swerdlow, D.L. (2010). Epidemiology of Seafood-Associated Infections in the United States. Clinical Microbiology Reviews, 23, 399-411.

Jacobson, M.Z. (2005). Studying ocean acidification with conservative, stable numerical schemes for nonequilibrium air-ocean exchange and ocean equilibrium chemistry. Journal of geographical research. 110, D70302.

Johnson, C.N., Bowers, J.C., Griffitt, K.J., Molina, V., Clostio, R.W., Pei, S., Laws, E., Paranjpye, R.N., Strom, M.S., Chen, A., Hasan, N.A., Huq, A., Noriea, N.F., Grimes, J, Colwell, R.R. (2010). Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the Coastal and Estuarine Waters of Louisiana, Maryland, Mississipi, and Washington (United States). Applied and Environmental Microbiology, 78, 7249-7257.

Johnson, W.G., Salinger, A.C., King, W.C. (1973). Survival of *Vibrio parahaemolyticus* in oyster shellstock at two different storage temperatures. Appl. Microbiol., 26, 122-123.

Kaneko, T., Colwell, R.R. (1973). Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. Journal of Bacteriology., 113, 24-32.

Kaneko, T., Colwell, R.R. (1975). Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. Appl. Microbiol, 30, 251-257.

Kaysner, C.A., DePaola, A. (2001). Ourbreaks of *Vibrio parahaemolyticus* gastroenteritis from raw oyster consumption: assessing the risk of consumption and genetic methods for detection of pathogenic strains. J. Shellfish Res., 19, 657.

Kural, A.G., Shearer, A.E.H., Kingsley, D.H., Chen, H. (2008). Conditions for high pressure inactivation of *Vibrio parahaemolyticus* in oysters. International Journal of Food Microbiology, 127, 1-5.

Lee R., Lovatelli, A., Ababouch, L. (2008). Bivalve depuration: fundamental and practical aspects. FAO. Fisheries Technical Paper 511.

Lee, C., Chen, H., Chuang, M. (1996). Use of oyster, *Crassostrea gigas*, and ambient water to assess metal pollution status of the charting coastal area, Taiwan, after the 1986 green oyster incident. Chemosphere, 33, 2505-2532.

Leong, Y.H., Gan, C-Y. (2014). Dioxin-Like Polychlorinated Biphenyls, Polychlorinated Dibenzop-Dioxins, and Polychlorinated Dibenzofurans in Seafood Samples from Malaysia: Estimated Human Intake and Associated Risks. Arch Environ Contam Toxicol. 67: 21-28.

Lipp, E.K., Rose, J.B. (1997). The role of seafood in foodborne diseases in the United States of America. Rev. sci. tech. Off. Int. Epiz., 16, 620-640.

Liu, C., Lu, J., Su, Y. (2009). Effects of flash freezing, followed by frozen storage, on reducing *Vibrio parahaemolyticus* in Pacific raw oysters (*Crassostrea gigas*). Journal of Food Protection, 72, 174-177.

Liu, D., Ning, X., Zhang, J. (2008). Optimization of growth condition of *Vibrio parahaemolyticus* via surface response methodology. Microbiology, 35, 306-310.
Liu, X., Chen, Y., Wang, X., Ji, R. (2004). Foodborne disease outbreaks in China from 1992 to 2001 national foodborne disease surveillance system. Wei Sheng Yan Jiu., 33, 725-727.

Loosanoff, V.L. (1958). Some aspects of behavior of oysters at different temperatures. Biol bull, 114, 57-70.

Loosanoff, V.L., Tommers, F.D. (1947). Effect of low pH upon the rate of water pumping of oysters, *Ostrea virginica*. Anat Rec, 99, 112-113.

Lozano-Leon A., Torres J., Osorio C.R., Martinez-Urtaza J. (2003) Identification of tdh-positive *Vibrio parahaemolyticus* from an outbreak associatated with raw oyster consumption in Spain. FEMS Microbiology Letters., 226, 281-284.

Ma, L., Su, Y-C. (2011) Validation of high pressure processing for inactivating *Vibrio parahaemolyticus* in Pacific oysters (Crassostrea gigas). International Journal of Food Microbiology., 144, 469-474.

Mahmoud, B.S.M. (2014). The efficacy of grape seed extract, citric acid and lactic acid on the inactivation of *Vibrio parahaemolyticus* in shucked oyster. Food Control. 41, 13-16.

Mahmound, B.S.M., Burrage, D.D. (2009). Inactivation of *Vibrio parahaemolyticus* in pure culture, whole live and half shell oysters (*Crassostrea virginica*) by X-ray. Applied Microbiology, 48, 572-578.

McLaughlin, J.B., DePaola, A., Bopp, C.A., Martinek, K.A., Napolilli, N.P., Allison, C.G., Murray, S.L., Thompson, E.C., Bird, M.M., Middaugh, J.P. (2005). Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. The New England Journal of Medicine., 353, 1463-1470.

Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K. and Yamai, S. (1969). In vitro hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. Journal of Bacteriology, 100, 1147-1149.

Miyamoto, Y., Obara, Y., Nikkawa, T., Yamai, S., Kato, T., Yamada, Y., Ohashi, M. (1980). Simplified purification and biophysicochemical characteristics of Kanagawa phenomenon-associated hemolysin of *Vibrio parahaemolyticus*. Infect. Immon., 28, 567-576.

Molenda, J.R., Johnson, W.G., Fishbein, M., Wentz, B., Mehlman, I.J., Dadisman, T.A. (1972). *Vibrio parahaemolyticus* gastroenteritis in Maryland: laboratory aspects. Appl. Microbiol 24: 444-448.

Morrissey, T.M. (2004). Issues regarding mercury in Pacific Northwest seafood. Sea Grant Program. www.oregonschoice.com/*mercury*.htm

Nishina, T., Wada, M., Ozawa, H., Hara-kudo, Y., Konuma, H., Hasegawa, J., Kumaga, S. (2004). Growth kinetics of *Vibrio parahaemolyticus* O3:K6 under varying conditions of pH, NaCl concentration and temperature. J. Food Hyg Soc. Japan, 45, 35-37.

[NSSP] National Shellfish Sanitation Program (2003). Guide for the Control of Molluscan Shellfish. Washington D.C.: Public Health Services, U.S. Food and Drug Administration. http://www.sfsan.fda.gov/~acrobat/nssp2003.pdf

[NSSP] National Shellfish Sanitation Program (2011). National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish: Section III. 197-200.

[NSSP] National Shellfish Sanitation Program (2011). National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish: Section IV. 329-336.

Parveen, S., DaSilva, L., DePaola, A., Bowers, J., White, C., Munasinghe, K.A., Brohawn, K., Mudoh, M., Tamplin, M. (2013). Development and validation of a predictive model for the growth of *Vibrio parahaemolyticus* in post-harvest shellstock oysters. International Journal of Food Microbiology. 161: 1-6.

Parveen, S., Hettiarachchi, K.A., Bowers, J.C., Jones, J.L., Tamplin, M.L., McKay, R., Beatty, W., Brohawn, K., DaSilva, L.V., DePaula, A. (2008). Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. International Journal of Food Microbiology, 128, 354-361.

Phuvasate, S., Su. Y. (2013). Impact of water salinity and types of oysters on depuration for reducing *Vibrio parahaemolyticus* in Pacific oysters (*Crassostrea gigas*). Food Control, 32, 569-573.

Potasman, I., Paz, A., Odeh, M. (2002). Infectious outbreaks associated with bivalve shellfish consumption: A worldwide perspective. CID, 35, 921-928.

Prato, R., Lopalco, P.L., Chironna, M., Barbuti, G., Germinario, C., Quarto, M. (2004). Norovirus gastroenteritis general outbreak associated with raw shellfish consumption in South Italy. Infectious Diseases, 4, 37-42.

Rippey, S.R. (1994). Infectious diseases associated with molluscan shellfish consumption. Clin. Microbiol. Rev., 7, 419-425.

Robert-Pillot, A., Guenole, A., Lesne, J., Delesmont, R., Fournier, J.M., Quilici, M.L. (2004). Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from

waters and raw shellfish collected in two French coastal areas and from seafood imported into France. International Journal of Food Microbiology. 91, 319-325.

Rowse, A.J., Fleet, G.H. (1984). Effects of water temperature and salinity on elimination of *Salmonella* charity and *Escherichia coli* from Sydney rock oysters (Crassostrea commercialis). Applied and Environmental Microbiology, 48, 1061-1063.

Safe, S. (1992). Toxicology, structure-function relationship, and human and environmental health impacts of polychlorinated biphenyls: progress and problems. Environmental health perspectives. 100, 259-268.

Sakazaki, J., Matsuzaki, A., Miwatani, T. (1973). Purification and characterization of thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immune., 8: 775-780.

Sakazaki, R. (1979). *Vibrio* infections. In: Riemann H and Bryan FL, editors. Food-Borne Infections and Intoxications. New York: Academic Press. 173-209.

Sakazaki, R. (1983). *Vibrio parahaemolyticus* as a food-spoilage organism. In: Rose AH, editor. Food Microbiology. New York: Academic Press. 225-241.

Sakazaki, R., Tamura, K., Kato, T., Obara, Y., Yamai, S., Hobo, K. (1968). Studies of the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. III. Enteropathogenicity. Jpn J Med Sci Biol, 21, 325-331.

Serment-Moreno, V., Deng, K., Su, Y.-C.,Wu, X., Fuentes C., Torres J.A., Welti-Chanes J. 2015. Monte Carlo analysis of the product handling and high-pressure treatment effects on the *Vibrio vulnificus* risk to raw oysters consumers. Journal of Food Engineering, 144, 86-92.

Shirai, H., Ito, H., Hirayama, T., Nakamoto, Y., Nakabayashi, N., Kumagai, K., Takeda, Y., Nishibuchi, M. (1990). Molecular epidemiologic evidence for association of Thermostable Direct Hemolysin (TDH) and TDH-Related Hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. Infection and Immunity, 58, 3568-3573.

Steuerwald, U., Weihe P., Jorgensen, P.J., Bjerve, K., Brock, J., Heinzow, B., Budtz-Jorgensen, E., Grandjean, P. (2000). Maternal seafood diet, methylmercury exposure, and neonatal neurologic function. The Journal of Pediatrics, 136, 599-605.

Storelli, M.M. (2008). Potential human health risks from metals (Hg, Cd, and Pb) and polychlorinated biphenyls (PCBs) via seafood consumption: Estimation of target

hazard quotients (THQs) and toxic equivalents (TEQs). Food and Chemical Toxicology, 46, 2782-2788.

Storelli, M.M., Giacominelli-Stuffler, R., Storelli, A., Marcotrigiano, G.O. (2003). Polychlorinated biphenyls in seafood: contamination levels and human dietary exposure. Food Chemistry, 82, 491-496.

Su Y., Yang Q., Hase, C. (2010). Refrigerated seawater depuration for reducing *Vibrio parahaemolyticus* contamination in Pacific oyster (*Crassostrea gigas*). Journal of Food Protection, 73, 1111-1115.

Su, Y., Liu, C. (2007). *Vibrio parahaemolyticus*: A concern of seafood safety. Food Microbiology, 24, 549-558.

Tamplin, M.L., Capers, G.M. (1992). Persistence of *Vibrio vulnificus* in tissues of Gulf Coast oysters (*Crassostrea virginica*), exposed to seawater disinfected with UV light. Appl Environ Microbiol, 58, 1506-1510.

Tang, Y.W., Wang, J.X., Xu, Z.Y., Guo, Y.F., Qian, W.H., Xu, J.X. (1991). A serologically confirmed, case-control study, of a large outbreak of hepatitis A in China, associated with consumption of clams. Epidemiol Infect., 107, 651-657.

Teegarden, G.J., Cembella, A.D. (1996). Grazing of toxic dinoflagellates, *Alexandrium* spp., by adult copepods of coastal Maine: Implications for the fate of paralytic shellfish toxins in marine food webs. Journal of Experimental Marine Biology and Ecology, 196, 145-176.

Wang, C., Wang S., Chang, T., Shi, L., Yang, H., Shao, Y., Feng, W., Cui, M. (2013). Efficacy of lactic acid in reducing foodborne pathogens in minimally processed lotus sprouts. Food Control, 20, 721-726.

Wang, X., Sato, T., Xing, B., Tao, S. (2005). Health risks of heavy metals to the general public in Tianjin, China via consumption of vegetables and fish. Science of the Total Environment, 350, 28-37.

[WHO] World Health Organization. (2004). In report: Persistent organic pollutants (POPs). Available at: http://www.who.int/foodsafety/areas_work/chemical-risks/pops/en/

[WHO] World Health Organization. (2010). Safe Management of Shellfish and Harvest Waters. Edited by Rees G., Pond K., Kay D., Bartram J., Domingo J.S.

[WHO] World Health Organization. (2014). In report: Dioxins and their effects on human health. Available at: http://www.who.int/mediacentre/factsheets/fs225/en/#

# Appendices

Appendix A. Reductions of *Vibrio parahaemolyticus* in Pacific oysters after 4 and 5 days of depuration in artificial seawater (30 ppt) at 12.5°C with an oyster to water ratio of 1:2 (number of oyster : liters of water)

| Days | Populations of *V. parahaemolyticus* in oysters (log MPN/g) |
|---|---|
| 0 | 5.55±0.15A |
| 1 | |
| 2 | |
| 3 | |
| 4 | 1.80±0.31B (3.75) |
| 5 | 1.55±0.61B (4.00) |

[a] Data are means of five determination ± standard deviation. Means with different letters in the same column are significantly different ($p<0.05$).
[b] Reductions in bacterial population (log MPN/g).