AN ABSTRACT OF THE THESIS OF

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Title: Further Optimization of Controlled Depuration for Eliminating *Vibrio parahaemolyticus* from Raw Oysters for Safe Consumption.

Abstract approved:

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Vibrio parahaemolyticus infections in the United Stated have been linked to consumption of raw shellfish, particularly oysters, with symptoms of headache, abdominal pain, nausea, diarrhea, vomiting. Depuration, as a post-harvest process, has a long history of being applied in shellfish industry to reduce sewage bacteria. In order to reduce the risks of *V. parahaemolyticus* infection associated with raw oyster consumption, FDA (2015) requires that post-harvest processes reduce *V. parahaemolyticus* and *V. vulnificus* to non-detectable levels (<30 MPN/g) and achieve a 3.52 log reduction.

Previous investigations have indicated that a refrigerated seawater depuration process at 12.5°C could significantly reduce *V. parahaemolyticus* contamination in

Pacific oysters; however, further optimization is necessary to achieve the regulatory target of >3.52 log MPN/g. The aim of this study was to investigate several factors, including flow rate and feeding status, to improve the efficacy of depuration in decreasing *V. parahaemolyticus* in oysters. The long-term goal for this research is adoption of depuration by the shellfish industry to produce safe oysters for raw consumption. Pacific oysters (*Crassostrea gigas*; n = 35 per trial) were inoculated with a cocktail of *V. parahaemolyticus* (10290, 10292,10293, BE 98-2029, and 027-1c1) in freshly prepared artificial seawater (70 L). Depuration was conducted on inoculated oysters with flow rates of 15, 20, 25 and 35 L/min at 12.5°C for up to 5 days. *V. parahaemolyticus* contamination was determined using a three-tube most probable number (MPN) method. The efficacy of microbial reductions of *V. parahaemolyticus* was significantly enhanced when the flow rate increased from 15 to 35 L/min. Depuration with a lower flow rate (15 L/min) for 5 days resulted in 2.39 log (MPN/g) reduction of *V. parahaemolyticus* in oysters, while depuration with higher flow rates of 20 and 25 L/min reached 2.73 and 2.80 log (MPN/g) reductions, respectively. Further increase in flow rate to 35 L/min resulted in an average reduction of 3.39 log MPN/g of *V. parahaemolyticus* in oysters after 5 days. These results suggest that depuration efficacy can be enhanced by further increasing the flow rate of the system.

Studies of application of algae treatment in oyster depuration revealed that addition of algae mixture in artificial seawater (ASW) for depuration did not

significantly impact the efficacy of depuration for reducing *V. parahaemolyticus* populations. Oysters inoculated with the *V. parahaemolyticus* cocktail were subjected to depuration with and without feeding (algae=0.036 ml/gram of oyster) for 6 days at 12.5°C with water ratio of 1:2. Oysters (n = 5) were analyzed for *V. parahaemolyticus* using a three-tube most probable number (MPN) method after 0, 1, 3, 5, and 6 days of depuration. Depuration over 6 days achieved average *V. parahaemolyticus* reductions of 2.75 log MPN/g and 3.03 log MPN/g in the fed and unfed systems, respectively. The lack of impact of feeding status on the efficacy of depuration provides the oyster industry with the flexibility to utilize fed or unfed conditions to apply in depuration process.

In these studies, only the factor of flow rate had a positive impact on reduction of *V. parahaemolyticus*. However, neither flow rate nor feeding treatments were able to achieve 3.52 MPN/g reduction of *V. parahaemolyticus*. Further optimization of depuration is necessary to achieve the regulatory target for *V. parahaemolyticus* decontamination in raw oysters.

Further Optimization of Controlled Depuration for Eliminating *Vibrio parahaemolyticus* from Raw Oysters for Safe Consumption

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Ziwen Ming, Author

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Chapter 1 General Introduction

1.1 Introduction of Shellfish

In recent years, world production and consumption of shellfish (oyster, mussel, clam, and scallop) has grew from a combined total of wild catch and aquaculture of approximately 13 million tons in 2009 to 16 million tons in 2015 (FAO, 2017). In 2015, shellfish farming accounted for nearly 21% of the total fish farming production for food consumption throughout the world (FAO, 2015). The U.S. oyster industry yielded 14 thousand tons of oysters which valued at over $213.8 million while the 4.8 million ton of total fish production valued $5.2 billion in 2015 (Pritzker et al., 2016). The world production of oyster in 2015 reached around 5.5 million tons which valued $4.2 billion (FAO, 2017). The top producing species is Eastern oyster (*Crassostrea virginica*) from the Gulf of Mexico which accounts for 78% of the national total. Pacific oyster (*Crassostrea gigas*) is the second major oyster species (21.5%) harvested in the U.S (Banks et al., 2007).

Shellfish, including oysters, are filter-feeders which affect water quality by ingesting the edible particles and expelling particles with mucus, then depositing this as pseudofeces onto the sediment surface (Zu Ermgassen et al., 2013). Biological characteristics of oyster feeding and filtration is well studied, and filtration rate is known to be influenced by environmental factors such as

temperature, flow rate, salinity, and particle size, and differs between oyster species. The Eastern oyster (*Crassostrea virginica*) is capable of filtering particles >5 μm in size with high efficiency (6.79 L/h) (Riisgård, 1988). Temperature is the most critical factor affecting the pumping rate. The maximum average pumping rate of oysters could be reached as high as 13 L/h at temperature of 28-30°C, while the lower temperature at 12-14°C produced the pumping rate of 5.4 L/h with average level of 3.9 L/h (Loosanoff, 1958). This physiology of oysters enables them to help with water clarity, but it can also lead to the accumulation of contaminants in their tissues. Ventilation, defined as the water flow rate over gills, leads to accumulation of bacteria on gills. Filtration (volume of water cleared per unit time) completed by sorting particles as food and sending them to the digestive tract contributes to accumulation of contaminants in the digestive tract of the oysters (Kennedy et al., 1996). It has been shown that around 70% of total *V. vulnificus* found in oyster meat is present in the gut, with less than 10% found in mantle and gills after 3 days of depuration at ambient temperature (Tamplin & Capers, 1992).

1.1.1 Bacterial hazards associated with shellfish/oysters

Naturally occurring bacteria including pathogenic and non-pathogenic *Vibrio* species, and bacteria from sewage contaminated environment including *Salmonella spp., Shigella spp*. and *Escherichia coli* are two broad groups of bacteria which will

mainly contaminate shellfish (FDA, 2015; Reilly & Käferstein, 1997). It was found that shellfish in U.S., particularly oysters, had a 1.2% prevalence of *Salmonella* (Heinitz et al., 2000). Fecal coliforms have been used as an indicator of other bacterial pathogens; however, research has demonstrated that *Salmonella* was present in oysters that did not contain high levels of fecal coliforms (Hood et al., 1983). According to FDA's requirement, harvesting water need to be tested per year for fecal coliforms. If fecal coliform levels exceed 230 MPN/g of oyster or 230 MPN/ml of water, then oyster systems are closed to harvesting (Andrews, 1995; FDA, 2015).

1.2 Introduction of *Vibrio parahaemolyticus*

1.2.1 Distribution of *V. parahaemolyticus*

The seasonal and geographical distributions of *V. parahaemolyticus* relate *V. parahaemolyticus* in both seawater and shellfish to temperature (DePaola et al., 1990). Isolation of *V. parahaemolyticus* from the environment has been shown to be related to water temperature, geographical, fecal pollution, season (rainy or dry) and salinity (DePaola et al., 2003; Molitoris et al., 1985). It is reported that *V.parahaemolyticus* was able to survive at temperature below 15°C in seawater (McLaughlin et al., 2005). Kaneko and Colwell (1973) reported that 10°C is a minimum temperature for growth of *V. parahaemolyticus* in the natural

environment. According to their research, it is uncommon to isolate *V. parahaemolyticus* during winter months in the Rhode River area of Chesapeake Bay, and a low rate of occurrence of *V. parahaemolyticus* infection was observed in winter months. Meanwhile, the population of *V. parahaemolyticus* increased to 10^4 CFU/100 ml from 10^2 CFU/100 ml when water temperature rose to 30°C in summer from 6°C in mid-winter (Kaneko & Colwell, 1973). In Indonesia, *V. parahaemolyticus* strains were isolated from seawater at temperatures ranging between 29°C and 32°C throughout the year (Molitoris et al., 1985). A study shows that between November 2002 and October 2003, there was a positive relationship observed between populations of *V. parahaemolyticus* in seawater and water temperature on the Oregon coast, and the highest populations (1100 MPN/g) of *V. parahaemolyticus* in water was detected in the summer months (Duan & Su, 2005). From November 2004 to October 2005, Parveen et al. (2008) found a significant positive association between population of *V. parahaemolyticus* and water temperature in Chesapeake Bay and water temperature is a major factor influencing the abundance of *V. parahaemolyticus* in seawater in temperate climate.

The population of *V. parahaemolyticus* in shellfish is also related to water temperature and is usually higher at warmer months. It has been reported that 29% of oysters harvested from the Pacific Northwest yielded *V. parahaemolyticus* only during the summer months when water temperatures ranged from 15°C to 22°C (Kelly & Stroh, 1988). Shellfish from the Gulf Coast typically had higher densities

of *V. parahaemolyticus* than did shellfish harvested from the North Atlantic or mid-Atlantic coast (Cook et al., 2002a). The mean density of *V. parahaemolyticus* in the Gulf Coast water at 22°C is around 1.1×10^4CFU/100 ml, while the populations of *V. parahaemolyticus* in the Atlantic at 15°C and in the Pacific at 17°C are 3×10^3 CFU/100 ml and 2.1×10^3 CFU/100 ml, respectively (DePaola et al., 1990). Parveen et al. (2008) Indicated that about 63% of the oyster samples from Chesapeake Bay containing detectable levels (10 CFU/g) of *V. parahaemolyticus* were collected at water temperature of > 14°C from April 2005 to October 2005.

The relationship between the population of *V. parahaemolyticus* and water salinity is unknown. Densities of *V. parahaemolyticus* were found to be positively correlated with water temperature, but not with water salinity or the overall bacterial population in seawater. Comparing higher water salinities from June to November with lower water salinities from December to May in Oregon Coast, Duan and Su found no correlation between water salinity and *V. parahaemolyticus* density (Duan & Su, 2005). However, higher *V. parahaemolyticus* densities were found at lower seawater salinity in the Smith Point and East Bay area (DePaola et al., 2000). In the Rias of Galicia, Spain, 61 positive samples of seawater (18%) had relatively high level populations of *V. parahaemolyticus* (1.2×10^3 MPN/100 g) and

were primarily detected during periods of lower salinity in autumn months (Martinez-Urtaza et al., 2008).

1.2.2 Incidence of *V. parahaemolyticus* Infection

V. parahaemolyticus was firstly reported as causing gastrointestinal episodes in Japan following the consumption of shirasu (semi-dried salted sardine) in 1950, which caused 272 cases with 20 deaths (Fujino et al., 1953). In the following years, *V. parahaemolyticus* becomes one of the most significant food-borne pathogens in Asia, causing approximately half of the food poisoning outbreaks in Taiwan, Japan, and Southeast Asian countries (Joseph et al., 1982). In Vietnam, 548 cases of *V. parahaemolyticus* infection were reported between 1997 and 1999 (Tuyet et al., 2002). From 2003 to 2008, 322 gastroenteritis outbreaks due to *V. parahaemolyticus* across 12 provinces in China with 9041 illnesses and 3948 hospitalizations were reported with 28% involving aquatic products (Wu et al., 2014). Strains of the O3:K6 serovar appeared in Calcutta, India, accounting for 50 to 80% of the *V. parahaemolyticus* strains isolated during the high-incidence period (February to August) in 1996 (Okuda et al., 1997). The incidence of diarrhea due to O3:K6 strains remains high: 63% of the *V. parahaemolyticus* strains isolated from patients in Calcutta between September 1996 and April 1997 belonged to the O3:K6 serovar and possessed the *tdh* gene alone.

V. parahaemolyticus was first identified as the etiological agent of gastroenteritis in U.S. after three outbreaks of 425 cases in Maryland in 1971 (Molenda et al., 1972). During a 3-month period in the late summer and fall of 1981, six cases of gastroenteritis and one wound infection due to *V. parahaemolyticus* were reported to public health agencies in Washington and Oregon. All the gastroenteritis illnesses were associated with eating raw oysters (Nolan et al., 1984). In summer of 1997, the larger outbreak of culture-confirmed *V. parahaemolyticus* infections happened in North America, involving 209 illnesses who were associated with eating raw oysters harvested from California, Oregon, and Washington in the United States and from British Columbia (BC) in Canada (CDC, 1998). During May 20--July 31, 2006, health departments of New York City, New York state, Oregon, and Washington reported an outbreak with a total of 177 cases of *V. parahaemolyticus* infection in which 122 cases have been associated with 17 seafood clusters (CDC, 2006). Most infections are associated with ingestion of raw or undercooked shellfish harvested from both the Gulf of Mexico and the Pacific Ocean (Cook et al., 2002b). There was a relative large outbreak in the Pacific Northwest in the summer of 1998, affecting 209 people, including one death (CDC, 1998). The largest outbreak of *V. parahaemolyticus* (O3 : K6 serotype) infection was reported in the United States in 1998, indicating 416 persons in 13 states having gastroenteritis after eating oysters harvested from Galveston Bay, Texas (N. A. Daniels, 2000). In 2013, serotypes O4: K12 and O4:

KUT strains traced from shellfish consumed by ill persons in U.S. Atlantic Coast caused illness in 104 persons from 13 states during May to September (Newton et al., 2014).

1.2.3 Virulence Factors and Symptoms of *V. parahaemolyticus*

It is believed that most strains of *V. parahaemolyticus* isolated from the environment or seafood are not pathogenic (Nishibuchi & Kaper, 1995). Clinical strains of *V. parahaemolyticus,* carrying the *tdh* gene, have ability to produce a thermostable direct hemolysin (TDH), which can lyse red blood cells on Wagatsuma blood agar plates (Joseph et al., 1982; Nishibuchi & Kaper, 1995). The hemolytic activity of TDH, called the Kanagawa phenomenon (KP), has been reported to be mostly found positive in patients with gastroenteritis but rarely KP-positive observed in environmental isolates (Joseph et al., 1982). However, in 1985, outbreak of gastroenteritis in Republic of Maldives isolated KP^- *V.parahaemolyticus* strains (Honda et al., 1988).This isolate cannot produce TDH but carry the *trh* gene which encodes TDH-related hemolysin. In Japan, it was reported that in 214 clinical strains of *V. parahaemolyticus*, 112 strains (52.3%) contained the *tdh* gene, 52 strains (24.3%) had the *trh* gene only, and 24 strains (11.2%) carried both the tdh and the trh gene (Shirai et al., 1990). The pathogenicity of *V. parahaemolyticus* appears to be associated with the presence of both *tdh* and *trh* genes (Su & Liu, 2007).

Symptoms of *V. parahaemolyticus* infection usually occur within 24 hours or ranging from 4 to 96 hours, after ingesting the bacteria. Typical symptoms of *V. parahaemolyticus* include diarrhea, nausea, vomiting, headache, fever, chills and abdominal pain (CDC, 2013). Most cases of infection usually include acute self-limiting diarrhea. Wound infection and septicemia caused by exposure to *V. parahaemolyticus* have also been reported (Qadri et al., 2003). The mortality rate with approximately 1–4% even existed in patients who develop septicemia (Butt et al., 2004). An infectious dose of *V. parahaemolyticus* is reported to be 10^5 CFU/g or more (Butt et al., 2004). Relatively low probability of illness (<0.001%) was observed in consumption of 50 cells/gram oysters. Consumption of 5×10^5 cells/gram oysters of *V. parahaemolyticus* raises the probability of illness to around 50% (FDA, 2005).

1.2.4 Factors affecting *V. parahaemolyticus* growth and survival

Growth and survival of *V. parahaemolyticus* are mainly influenced by several factors: temperature, salinity, water activity, and pH. Given the rapid growth rate of this organism, the optimum temperatures for growth and survivals of *V. parahaemolyticus* are between 35°C to 37°C (Beuchat, 1975). Maximum temperatures for growth of *V. parahaemolyticus* range from 42°C to 44°C. Lowest temperatures reported are highly dependent on the growth substrate and habitat, usually ranging from 3°C to 13°C (Lee, 1973). It is not uncommon to isolate *V.*

parahaemolyticus from oysters and marine sediment samples below 10°C. However, *V. parahaemolyticus* are more commonly isolated from seawater when the water temperature is above 13°C (Molenda et al., 1972).

The halophilism is one of important characteristics of this organism, early recognized by the Japanese investigators, and thus a new agent of food poisoning organism was established (T. Fujino, 1964). *V. parahaemolyticus* requires sodium chloride for growth in substrates containing as little as 0.5% at normal growing temperature (Beuchat, 1975). Lee reported that the maximum sodium chloride concentration tolerated by *V. parahaemolyticus* is 8% (Lee, 1973). On the other hand, the tolerance of *V. parahaemolyticus* to sodium chloride concentrations is also related to osmotic and ionic sensitivity. Once the organism is exposed in distill water, it is easily inactivated with 90% death within 0.9 to 4.4 min (Lee, 1972).

V. parahaemolyticus always grows well under alkaline pH. The recommended pH for the culture media is from 7.4 to 8.6 (Lee, 1973). In the study of Kodama, foods with pH below 5.8 do not support growth of *V. parahaemolyticus* and maximum growth was demonstrated in uncooked octopus and marinated egg with respective pH values at 7.7 and 8.5 (Kodama, 1967).

The different factors of temperature, salt concentration and pH also have cross impact on growth and survival of *V. parahaemolyticus*. Beuchat (1973) tested six strains of *V. parahaemolyticus* at different temperature, salt concentrations in media of different pH and reported an increasing tendency of growth at lower pH as the

incubation temperature was increased. Growth at 5°C and 9°C was only shown at an alkaline pH. In his study, two strains, 107914 and 8700, had the highest tolerance to low pH (4.8) and grew at 30°C with 3% NaCl. Growth of *V. parahaemolyticus* in different NaCl concentrations can greatly affect its response to pH and temperature stresses (Beuchat, 1973). Whitaker et al. investigated their ability to grow at different salt concentrations under different other environmental stresses like temperature and pH fluctuations. When the NaCl concentration of the medium was reduced to 0.5% NaCl, *V. parahaemolyticus* failed to grow at pH 5 but grew better with 3% NaCl. They also found that *V. parahaemolyticus* grown in 3% NaCl have higher tolerance to temperature stress (42^{o}C) than cells grown in 1% NaCl (Whitaker et al., 2010).

1.2.5 Detection of *V. parahaemolyticus*

Selective enrichment broth and agar media are developed to detect and isolate *V. parahaemolyticus* in foods. Selective enrichment of alkaline peptone water (APW), salt polymyxin broth (SPB) and plating of the enrichment culture onto thiosulfate citrate bile salt sucrose (TCBS) agar have been widely used for selective isolation of *V. parahaemolyticus* from foods (Hara-Kudo et al., 2001). *Vibrio* species growth requires the presence of relatively high levels of bile salts, thus media used for testing the biochemical reactions of *V. parahaemolyticus* should contain 2% or 3% NaCl. Thiosulfate citrate bile salt sucrose (TCBS) agar is a

selective medium commonly used for isolating *V. cholerae, V. parahaemolyticus,* and other *Vibrio* species from seafood while inhibiting most non-vibrios (Kaysner & DePaola, 2004). Inhibition of Gram-positive bacteria is achieved by the incorporation of oxgall. Differentiation within *Vibrio* species is based on sucrose fermentation. *Vibrio cholerae* has the ability to ferment sucrose and produce acid to form yellow colonies, while *V. parahaemolyicus* and most *V. vulnificus* don't ferment sucrose and produce green colonies (Kobayashi et al., 1963). However, direct plating is efficient only when the concentrations of targeted bacteria are high in samples because the result will be affected if other bacteria exist at high concentration.

Most probable number (MPN) is a serial dilution test measuring the concentration of a target microbe in a sample with an estimate (Blodgett, 2010). Most common method for detection of *V. parahaemolyticus* is MPN method with selective enrichment of alkaline peptone water (APW) and plating of the enrichment culture onto TCBS agar.

In recent years, sensitive, rapid and accurate polymerase chain reaction (PCR) assays and real-time PCR have been developed for identification of *V. parahaemolyticus* by targeting one or more of the genes in different samples. A specific *toxR*-targeted PCR assay was shown to be a rapid and reliable method for *V. parahaemolyticus* identification(Kim et al., 1999). The real-time PCR were applied for detection of *V. parahaemolyticus* using a fluorescently labeled TaqMan

probe targeting a single locus (*tdh*) and dual loci in a multiplexed format (*tdh* and *toxR*) for pathogenic strains (Blackstone et al., 2003; Iijima et al., 2004). A conventional multiplexed PCR assay targeting *tlh* gene for total, and both *tdh* and *trh* genes of *V. parahaemolyticus* is necessary for comprehensive detection of this pathogen in shellfish (Bej et al., 1999; Ward & Bej, 2006).

In addition to traditional culture methods and PCR method, immunological techniques like enzyme-linked immunosorbent assays (ELISA) and immunochromatography have also been developed for simple and rapid analysis of *V. parahaemolyticus* (Su, 2012).

1.3 Preventions and Controls of *V. parahaemolyticus* Infection

It is already known that *V. parahaemolyticus* can reproduce rapidly to an infectious dose in seafood if temperature abused between harvest and consumption. Although it can be destroyed by adequate cooking, existence of V. *parahaemolyticus* in shellfish, especially for raw consumption, is still a safety issue (Phuvsate & Su, 2012). FDA established a guidance limit of 10,000 viable cells per gram for *V. parahaemolyticus* in ready-to-eat shellfish (FDA, 2011). Cook et al. studied that the population of *V. parahaemolyticus* or *V. vulnificus* of 10^5 cells/gram was readily reached in market oysters harvested from the Gulf Coast during summer months. A WHO/FAO risk assessment indicated that concentration below 30 cells/gram of *Vibrio* species is a negligible health risk. In order to ensure a post-

harvest process to be used throughout the year, the ISSC adopted a protocol to assure that the process should be able to reduce levels of *Vibrio* species from an initial MPN level of 100,000/gram to <30/gram (equivalent to a 3.52 Log reduction) (FDA, 2015).

To minimize the risk of growth of *Vibrio* spp., water quality of harvest area, post-harvest temperature, transportation and storage were regulated under National Shellfish Sanitation Program (NSSP). NSSP established temperature-time control to limit maximum hours from exposure after harvest to temperature control process (means of ice, mechanical refrigeration or other approved means capable of cooling shellstock to 50°F or less) according to local average monthly maximum air temperature (FDA, 2015). Shellfish harvested for raw consumption need to be cooled down to 10°C (50°F) within 12, 18, 24, and 36 h after harvest when the average monthly maximum air temperature is ⩾27 °C (81 °F), between 15 and 27 °C (60–80 °F), between 10 and 15 °C (50–60 °F), and <10 °C (50 °F), respectively (FDA, 2015). In addition to the above, control for *V. parahaemolyticus* in shellfish is also based on growing area. A post-harvest process which can eliminate *V. parahaemolyticus* to non-detectable level is needed if the growing area has an average monthly daytime water temperature that exceeds 60°F for waters bordering the Pacific Ocean or exceeds 81°F for waters bordering the Gulf of Mexico and the Atlantic Ocean (New Jersey and south) in harvesting time(FDA, 2015).

Tagging the oysters helps authorities to track down shellfish in the marketplace back to the distribution and to the harvester when an outbreak occurs. All raw or post-processed shellfish are required to have a tag bearing information, including the name of the location where the raw shellfish was harvested, harvest date, shucking date and sell-by-day.

1.4 Post-harvest process

Post-harvest processes(PHP) for shellfish are used to reduce naturally occurring pathogens in marine environments. They are not intended to remediate or remove pathogens associated with sewage pollution. FDA requires a valid post-harvest process must achieve a 3.52 log MPN/g reduction of *V. parahaemolyticus* and *V. vulnificus* in shellfish and to non-detectable levels (<30MPN/g) (FDA, 2015). Post-harvest processes including thermal treatment, quick frozen (IQF), high hydrostatic pressure (HHP), irradiation, and depuration may be valid options to eliminate *V. parahaemolyticus* in oysters.

1.4.1 Thermal Treatment

V. parahaemolyticus is sensitive to heat treatment. A survey from the U.S. Food and Drug Administration indicated that 90% of collected oysters subjected to mild heat post-harvest processing from June through October 2004 had 0.04 MPN/g of *V. parahaemolyticus* survivors, which is 750-fold lower than specified amount for

non-detectable levels (30 MPN/g) (DePaola et al., 2009). The use of low temperature pasteurization for raw shell-stock oysters at 50°C was very effective in reducing the pathogens *V. vulnifcus* and *V. parahaemolyticus* from 10^5 MPN/g to non-detectable levels in 10 min (Andrews et al., 2000). *V. parahaemolyticus* were found to be more heat resistant than the other *Vibrio* species and it needed heat treatment at 55°C for 9 min for 5-log reduction (Johnston & Brown, 2002). The author also suggested that higher temperature of pasteurization at 70°C for 2 min was effective against all *Vibrio* spp. Liu et al. (2010) indicated that heating process in hot water at 80 °C or higher for 1 min is able to reduce *V. parahaemolyticus* in the clams to non-detectable levels.

1.4.2 Refrigeration and freezing process

Vibrio spp. are sensitive to low temperatures and quality and safety of seafood are largely dependent on the temperature control (Baross & Liston, 1970). Storage at refrigeration temperatures below 5°C is able to inhibit growth of *Vibrio* spp. (Natarajan et al., 1980). Gooch et al. (2002) studied that *V. parahaemolyticus* increased rapidly by 2.9 log CFU/g in live oysters held at 26°C within 24 h, while a decrease of 0.8 log CFU/g was observed after approximately 14 days of refrigeration (3°C). In research of Phuvasate (2012), there was a similar small reduction (<0.8 log MPN/g) of *V. parahaemolyticusin* in oysters during four days of storage in ice. Moreover, keeping clams stored at 5°C and 0°C for 10 days

resulted in higher in reductions of *V. parahaemolyticus* (1.98 and 2.32 log MPN/g, respectively) (Liu et al., 2010). Shen et al. (2009) also reported that refrigerated storage at 5℃ and 0℃ can reduce populations of *V. parahaemolyticus* in shell stock by 1.42 and 2.11 log MPN/g, respectively only within four days. However, an earlier study determined that *V. parahaemolyticus* can survive at 4°C for at least 3 weeks with no apparent decrease in numbers (Johnson et al., 1973).

In frozen storage, *V. parahaemolyticus* was effectively inactivated in shell stock and shucked oysters by reaching >3.52 MPN/g after 75 days of storage at -30 ℃ (Shen et al., 2009). Similarly, Liu demonstrated that frozen storage of clam at -18℃ for 15 days or at -30℃ for 30 days can reduce *V. parahaemolyticus* from 4.05 log MPN/g to non-detectable levels. Flash freezing followed by frozen storage at -10, -20, and -30℃ decreased the populations of *V. parahaemolyticus* in the half-shell oysters by 2.45, 1.71, and 1.45 log MPN/g after 1 month of storage respectively, and storing oysters at −10℃ was more effective in inactivating *V. parahaemolyticus* than other frozen conditions (Liu et al., 2009). The survival of *V. parahaemolyticus* in fish fillet, picked crabmeat and oysters declined more rapidly at -15℃ after one month frozen storage than at −30℃ (Johnson & Liston, 1973). *Vibrio* spp. tend to survive better in frozen products stored at lower freezing temperatures because small bacterial cells form at lower temperatures are less damaged by ice crystals formed in frozen storage (Jay et al., 2005).

1.4.3 Irradiation

Irradiation including gamma irradiation, electron beam and X rays have been shown to be effective for controlling pathogens in seafood. Gamma rays are effective at reducing *Vibrio* spp. in artificially contaminated oysters with a dose of 1.2 kGy able to reduce *Vibrio* spp. as high as 10^7 CFU/g (Despaigne et al., 2001).

A dose of 1.0 kGy gamma radiation was sufficient to produce a 6-$\log_{10}$ reduction in the level of *V. parahaemolyticu*s. The highest irradiation dose did not kill the oysters nor affect their sensory attributes (Jakabi et al., 2003). To take advantage of physical property that the X-ray irradiator does not have a radioactive source (Anderson et al., 1989), X-irradiation is an alternative to gamma rays and has been used in food industry. In 2009, X-ray was first reported to describe inactivation of inoculated *V. parahaemolyticus* in oysters by X-ray (Mahmoud & Burrage, 2009). They found that X-ray (1-5 kGy) reduced the population of *V. parahaemolyticus* and inherent microflora in both half and whole shell oysters to non-detectable limit. *Vibrio* spp. is more sensitive to X-ray in pure culture than in whole oysters and a higher dose of X-ray is required to eliminate *V. parahaemolyticus* to non-detectable level in whole oysters (Mahmoud & Burrage, 2009). Mahmoud (2009) also found that *V. vunificus* requires lower dose of X-ray than *V. parahaemolyticus* to be reduced to non-detectable level. In a similar study, Andrews et al. (2003) reported that *V. vunificus* was more sensitive than *V. parahaemolyticus* to gamma irradiation.

1.4.4 High Pressure Processing (HPP)

High pressure processing (HPP) has been increasingly applied in industries as a processing method of reducing microorganism in oysters. *Vibrio* spp. are very sensitive to pressure compared than other bacteria. High pressure processing (200 to 350MPa; 5-15min; 25 °C) inactivated all strains of pathogenic *Vibrio* species in artificial seawater to non-detectable level (Berlin et al., 1999). In live oysters, Kural et al. (2008) reported that a 5-log reduction of *V. parahaemolyticus* can be achieved through ≥ 350 MP pressure treatment for 2 min at 1-35°C and ≥ 300 MPa for 2 min at 40°C. Cook (2003) compared the sensitivity of *Vibrio* species and found that *V. vulnficus* was most sensitive to HHP treatment and *V. cholerae* was most resistant to treatment. In their results, 250 MPa for 2 min achieved a >5 log reduction of naturally occurring *V. vulnficus* in oysters, while a 5-log reduction of *V. parahaemolyticus* serotype O3:K6 in oysters required a pressure of 300 MPa for 3 min. Similar research indicated that a HPP process of 293 MPa for 2 min at groundwater temperature (8 ± 1 °C) can produce greater than 3.52 log reductions of *V. parahaemolyticus* in Pacific oysters and oysters at this process maintained shelf life for 6-8 days stored at 5°C or 16–18 days stored in ice (Ma & Su, 2011).

1.5 Depuration

Depuration is a controlled process of holding oysters in a recirculating, sterilized seawater system and allowing them release contaminants from digestive

tract in clean seawater (Blogoslawski & Stewart, 1983). Depurated shellfish often command a premium price in the marketplace. Less gritty products due to purged sand, along with microbial and chemical contaminants, from the shellfish during the depuration lead to higher palatability to consumers (Richards, 1988). Depuration is based on the appropriate physiological conditions to ensure pumping activity of shellfish and effective depuration for contaminant removal occurs within a narrow range of conditions (Lee et al., 2008). *Salmonella, Escherichia coli,* and *Pseudomonas* spp. have been shown to be easily reduced in oysters depurated at ambient temperature, while reduction of *Vibrio* spp. requires longer periods of time (Croci et al., 2002; Son & Fleet, 1980; Vasconcelos & Lee, 1972). Kelly and Dinuzzo (1985) reported that it took 16 days to decrease *V. vulnificus* in artificial contaminated oysters to non-detectable level.

Several studies have been conducted to investigate optimal conditions on the efficacy of depuration in reducing *Vibrio* spp. levels in oysters. Four critical factors (water temperature, salinity, dissolved oxygen, and turbidity and suspended solids) affecting the physiological activity, pumping rate and behavioral responses of shell stock will directly influence the efficacy of depuration (FDA, 2015).

1.5.1 Factors effecting Depuration

1.5.1.1 Temperature

Water temperature is the most critical factor affecting efficacy of depuration because physiological activity of oysters and growth and survival of *V. parahaemolyticus* largely depend on temperature (Beuchat, 1975; FDA, 2015). Temperature is the most important factor associated with pumping rate of oyster (Loosanoff, 1958). The maximum average pumping rate of oysters could be reached as high as 13 L/h at temperature of 28-30°C (Loosanoff, 1958). It has been shown that viruses are removed much more slowly during depuration in Pacific oysters (*Crassostrea gigas*) than *E. coli*. Depuration at temperatures from 18°C to 21°C, resulted in more rapid efficiency of removing viruses from the shellfish, but it still took 5–7 days to achieve non-detectable levels at such temperatures (Lee et al., 2008).

A study on the elimination of *Vibrio* spp. and *E. coli* in a recirculating depuration system concluded that *E. coli* was rapidly eliminated at 8, 15 and 25°C, but the optimal temperature for reduction of *V. parahaemolyticus* was 15°C (Greenberg et al., 1982). Chae et al. (2009) reported that the survival of *V. parahaemolyticus* and *V. vulnificus* in American oysters (*Crassostrea virginica*) in artificial seawater during depuration at 15°C were reduced by 2.10 and 2.90 log MPN/g, respectively in two days, while a 1.10 log MPN/g reduction of *V. parahaemolyticus* was reached by depuration at 10 or 22°C. Depuration at reduced

temperatures (7-15°C, 5 days) has been shown to reduce *V. parahaemolyticus* in oysters by >3.0 log MPN/g (Phuvasate et al., 2012).

1.5.1.2 Salinity

Salinity is also an important factor on survival of shellfish and efficacy of depuration. The minimum salt concentration for the effective purification of *Crassostrea gigas* and *Crassostrea angulate* has been reported as 20.5 ppt (parts per thousand). The oysters suffered stress so that the self-purification was not effective at water salinities of 15 to 20 ppt, while a higher salinities of 32 to 47 ppt achieved rapidly purification (Rowse & Fleet, 1984). Reduction of salinity to 50 or 60% of the levels from which the shellfish were harvested prevented the depuration process (Liu et al., 1967). A previous study reported that optimal salinities for removing *E. coli* in depuration from hard clams were from 22 to 31 ppt (Heffernan & Cabelli, 1970). Presnell et al. (1969) investigated the salinity ranges for depuration efficiency of purging fecal coliforms in Eastern oysters and found that increasing salinities (24.8-25.5 ppt) of depurated seawater efficiently increases coliform reductions (99.8%). Similarly, a recent study indicated that depuration with a salinity of 10 ppt for 5 days resulted in ~2 $\log_{10}$ MPN/g reductions of *V. parahaemolyticus* in Pacific oysters, while a higher salinity (20–30 ppt) produce >3.0 $\log_{10}$ MPN/g reduction (Phuvasate & Su, 2013). In more recent study, high salinity (29-33ppt) in relaying process or recirculating aquaculture system help

to enhance the efficacy in reducing *V. vulnificus*, with reduction of 2 to 5 logs after 21 to 28 days, respectively (Parveen et al., 2017).

1.5.1.3 Flow rate

Flow rates also affected shellfish depuration. FDA requires a minimum flow rate of 107 liters per minute per cubic meter of shellfish for depuration system to maintain adequate oxygen levels (FDA, 2015). Heffernan and Cabelli (1970) reported that a flow rate of 13 ml/min/hard clam provided optimal depuration of *E. coli* and when the flow rate below 3 ml/min/clam, clams stopped pumping. The flow rates from 0.5-5.0 L/oyster/h can achieve >98% reduction of total and fecal coliforms at ambient temperature (Presnell et al., 1969). Lewis et al. (2010) demonstrated that increasing the flow rate (68 L/min) of the depuration system increased reductions (up to 4.56 Log CFU/g) of *V. vulnificus* in Eastern oysters, while depuration at lower flow rate of 11 L/min that even caused little increase in *V. vulnificus* population (1.4×10^3-2.4×10^3CFU/g).

1.5.1.4 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 and Tommers (1947) found that oysters could pump normally at pH 7.75, but the pumping rate dropped when pH decreased to 6.5. The oyster pumping rate declined to 10% of the normal

rate if pH was as low as 4.14. Turbidity and total suspended particles have a negative influence on oysters pumping ability and reduce the efficacy of ultraviolet light used to sterilize seawater in a depuration system (Richards, 1988). In addition, dissolved oxygen in seawater which supported the biological activity of oyster can also affect depuration.

1.6 Objective

Most post-harvest processes, including heating, low-temperature pasteurization, freezing and frozen storage, high pressure processing, and irradiation have been

investigated for reducing populations of *V. parahaemolyticus* in contaminated oysters. These processes have limitation for industry due to some negative effects like high costs or affecting quality of oysters. An economical and effective post-harvest process for reducing *V. parahaemolyticus* populations in live oysters to a safe level for raw consumption is needed.

Previous studies have found that depuration with artificial seawater at 12.5 ˚C for 5 days reduced *V. parahaemolyticus* populations in the Pacific oysters by > 3.0 log MPN/g. This study was conducted to investigate the different flow rates (15, 20, 25 and 35L/min) of depuration system on purging *V. parahaemolyticus* for potential application and if feeding treatment in depuration is able to increase the efficacy of depuration in decreasing *V. parahaemolytics*

Chapter 2 Flow rate of depuration system has minimal impact on *Vibrio parahaemolyticus* decontamination in Pacific Oysters (*Crassostrea gigas*)

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2.1 Abstract

Vibrio parahaemolyticus infections in the United Stated have been linked to consumption of raw oysters. Depuration has the potential to reduce contamination in live oysters after harvest. This study investigated the impact of depuration flow rate to reduce *V. parahaemolyticus* in raw oysters. Pacific oysters (n = 35 per trial) were inoculated with a cocktail of *V. parahaemolyticus* (10290, 10292,10293, BE 98-2029, and 027-1c1) in freshly prepared artificial seawater (70 L). The inoculated oysters were depurated with flow rates of 15, 20, 25 and 35 L/min at 12.5°C for up to 5 days and *V. parahaemolyticus* contamination was determined using a three-tube most probable number (MPN) method. *V. parahaemolyticus* reductions were as flow rate moderately increased from 15 L/min (2.39 log MPN/g reduction in 5 days) to 35 L/min (3.39 log MPN/g reduction). These results suggest that depuration efficacy can be enhanced by increasing depuration flow rate to 35 L/min.

Practical Applications

Vibrio parahaemolyticus can contaminate raw shellfish, including oysters, during their production and lead to outbreaks of foodborne illness. Depuration, a post-harvest process, may be used by the shellfish industry to reduce the persistence of *V. parahaemolyticus*. Previous studies have demonstrated that the depuration process can reduce *V. parahaemolyticus* in oysters; however, further optimization of the process is necessary to achieve FDA's targeted reduction goal (>3.52 log MPN/g). This study evaluated the impact of depuration flow rate on the reduction

of *V. parahaemolyticus* in Pacific oysters. Increasing flow rates (15 L/min to 35 L/min) during depuration enhanced the clearance of *V. parahaemolyticus* in these oysters; however, these conditions were unable to consistently achieve the target of >3.52 log MPN/g reduction. This study provides a reference for the industry on the variability of *V. parahaemolyticus* in individual oysters and demonstrates that practical modifications (i.e., flow rate) can be implemented in depuration systems to maximize bacterial clearance.

2.2 Introduction

Vibrio parahaemolyticus is frequently isolated from raw seafood, particularly oysters, and is the species most frequently associated with foodborne *Vibrio*-associated gastroenteritis in the U.S. (Bubb, 1975; DePaola et al., 1990). Symptoms of *V. parahaemolyticus* infection (headache, abdominal pain, nausea, diarrhea, vomiting) usually occur within 24 hours of consumption, but symptom onset may range from 4 to 96 hours (CDC, 2013). Most *V. parahaemolyticus* infections in U.S are associated with shellfish harvested from either the Gulf of Mexico or the Pacific Ocean (Cook et al., 2002b). The largest outbreak of *V. parahaemolyticus* in the U.S. occurred in 1998 with 416 persons in 13 states suffering gastroenteritis after eating oysters harvested from Galveston Bay, Texas (Daniels et al., 2000). A 2013 *V. parahaemolyticus* outbreak involving 104 individuals along the U.S. Atlantic Coast was traced to shellfish consumption (Newton et al., 2014).

Previous research has demonstrated the ability of *V. parahaemolyticus* to grow (up to 3 Log CFU/g increase in 24 h) in oysters during warm months (Gooch et al., 2001). To reduce the likelihood of infections of *Vibrio* spp. associated with raw shellfish consumption, the U.S. National Shellfish Sanitation Program (NSSP) limits the maximum time from harvest to refrigeration (≤10°C). Maximum allowable cooling time differs by region and is based on average maximum air temperature (FDA, 2015). While this approach will minimize the growth of *V. parahaemolyticus* in harvested oysters, it does not eliminate the risk of illness. Post-harvest processes, including thermal processing, cold storage, irradiation, and high pressure (HPP), have been demonstrated to reduce *V. parahaemolyticus* in oysters; however, these processes have a negative impact on oyster viability and product quality (Dionísio et al., 2009; Su, 2012). FDA defines effective post-harvest processes as those that reduce levels of *Vibrio* spp. from an initial MPN level of 100,000/gram to <30/gram (3.52 log MPN/g reduction) (FDA, 2015).

Oysters are filter-feeding animals which are capable of filtering large volumes (13 L/h) of seawater in relatively short periods of time (6 hours) (Loosanoff, 1958). Filter-feeding also enables oysters to release contaminants like bacteria (Chae et al., 2009; Sunnotel et al., 2007), viruses (Sobsey et al., 1987), and marine toxins (Cunningham & Tripp, 1973) into the bulk water. To take advantage of this biological process, depuration, a controlled process of holding oysters in a recirculating, sterilized seawater system, has been proposed as an option to reduce

V. parahaemolyticus contamination of raw oysters (Blogoslawski & Stewart, 1983). *Salmonella*, *Escherichia coli*, and *Pseudomonas* spp. have been shown to be easily reduced in oysters depurated at ambient temperature; however, *Vibrio* spp. persist in the oysters for longer periods of time (Son & Fleet, 1980; Vasconcelos & Lee, 1972). Depuration at reduced temperatures (7-15°C, 5 days) has been shown to reduce *V. parahaemolyticus* in oysters (Phuvasate et al., 2012); however, further optimization is necessary to achieve the FDA target of a 3.52 log CFU/g reduction.

Flow rates for depuration systems must be operated at minimal 107 liters per minute per cubic meter of shellfish to achieve oxygen levels necessary maintaining oyster viability following harvest (FDA, 2015). Previous research has demonstrated that increasing the flow rate of seawater has a positive effect on oyster filtering rate (Wilson-Ormond et al., 1997). Lewis (2010) demonstrated that increasing the flow rate (68 L/min) of the depuration system led to increased reductions (up to 4.56 Log CFU/g) of *V. vulnificus* in Eastern oysters. The objective of this study was to determine the influence of depuration flow rate on the reduction of *V. parahaemolyticus* in the Pacific oysters.

2.3 Material and Methods

2.4 *Vibrio parahaemolyticus* culture preparation

Five clinical strains of *V. parahaemolyticus* 10290 (serotype O4:K12, *tdh*+, *trh*+), 10292 (serotype O6:K18, *tdh*+, *trh*+), 10293 (serotype O1:K56, *tdh*+, *trh*+), BE 98-2029 (serotype O3:K6, *tdh*+, *trh*-), 1C1-O27 (serotype O5:K15, *tdh*+, *trh*-) were previously obtained from the FDA Pacific Regional Laboratory Northwest (Bothell, WA, USA). Strains were individually grown in 10 ml tryptic soy broth (Difco, Becton, Dickinson, Sparks, MD, USA) supplemented with 1.5% NaCl (TSB-Salt) at 35-37°C for 16-18 h. Each enriched culture was streaked onto a thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) plate and incubated at 37°C for 18-24 h. One single colony from each TCBS plate was transferred into 10 ml TSB-Salt and incubated at 35-37°C for 4 hr. Five enriched cultures of *V. parahaemolyticus* were then pooled into a 50-mL sterile centrifuge tube and harvested by centrifugation at 3000 x *g* at 5°C for 15 min. Pelleted cells were re-suspended in 2% salt solution to produce a culture suspension of 8-9 log CFU/ml.

2.4.1 Oyster inoculation with *V. parahaemolyticus*

Freshly harvested Pacific oysters (small size; ~30 grams/oyster) were collected from Washington oyster farm (Chetlo Harbor Shellfish, Ilwaco, WA) and transported on ice to the Seafood Laboratory (Astoria, OR). Artificial seawater

(ASW) was prepared by dissolving Instant Ocean salt (Aquarium systems Inc., Mentor, OH) in deionized water to achieve a salinity of 30 ppt (parts per thousand). Oysters were washed with tap water to remove mud and then placed in a HDPE tank (45 × 30 × 30 cm; Nalgene, Rochester, NY, USA) containing 20 L artificial seawater (ASW). Oysters were held under ambient conditions (~20°C) with air continuously pumped into the tank overnight to keep oxygen level favorable to maintain biological activities of oysters. For inoculation, oysters (n = 35) were then submerged in ASW (20 L) containing the *V. parahaemolyticus* cocktail at a cell density of ~5 log CFU/ml and held overnight as described above (Su et al., 2010).

2.4.2 Depuration treatment

Oysters were depurated in a laboratory-scale system composed of a 15 W Gamma UV sterilizer (Current-USA Inc., Vista, CA, USA), a water chiller (Delta star, Aqua Logic, Inc., San Diego, CA, USA), temperature controller (set at 12.5°C; Ranco ETC, Mexico), and a recirculating pump (Pan World Co., Ltd, Japan). Depuration process was conducted with 35 oysters in 70 L of ASW (oyster:water = 1:2) using flow rates of 15, 20, 25, or 35 L/min by different pumps for a maximum of 5 days.

2.4.3 Microbiological analysis.

Concentrations of *V. parahaemolyticus* in oysters were determined by using the three-tube most-probable-number (MPN) method (Kaysner & DePaola, 2004).

Oysters (n = 5-6/time point) were randomly selected for analysis at each time point (i.e., days 0, 1, 3 5). Individual oysters were transferred from the depuration tank, shucked with a sterile shucking knife, and the oyster meat was aseptically transferred to a sterile blender jar. Oyster meat was homogenized with an equal volume of sterile alkaline phosphate buffer saline (PBS; pH 7.4) for 1 min at high speed by a laboratory blender (Waring Laboratory, Torrington, CT). Twenty grams of homogenized oyster sample (1:2 dilution) was mixed with 80 ml of PBS to prepare 1:10 dilution sample suspension. Additional 10-fold dilutions of the sample suspension were prepared using PBS. Sample dilutions were individually inoculated into 3 tubes of alkaline peptone water (APW; pH 8.5; Difco, Becton Dickinson). APW tubes were incubated at 37°C for 16-18 h. 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) plates and incubated at 35-37°C for 18-24 h. Round, green or bluish colonies with 2-3 mm diameter on a TCBS plate after incubation was considered positive for *V. parahaemolyticus*.

2.4.4 Statistical analysis

Microbiological population levels were log-transformed and statistically analyzed using mixed model Analysis of Variance (ANOVA), and multiple-comparison of Tukey-Kramer using JMP (SAS Institute Inc, Cary, NC). Significant differences between reductions of *V. parahaemolyticus* at various times and flow

rates during depuration were established at $P < 0.05$. Linear fit was used to describe the rate of reduction over time.

2.5 Results and Discussion

Survivors of *V. parahaemolyticus* over time at 15 L/min is reported in Figure 2.1. Depuration at 15 L/min resulted in reduction of the average *V. parahaemolyticus* population from 4.93 log MPN/g to 2.54 log MPN/g in five days. There was a large variability in the results during the first three days of depuration, with day 3 exhibiting the widest range from 2.46 log MPN/g to 4.38 log MPN/g (standard error = 0.33). This range is likely due to differences in filtering rates and activity of individual oysters. Linear estimates of survivors indicated a reduction rate of 0.41 log MPN/g/day of *V. parahaemolyticus* in oysters using the 15 L/min flow rate ($R^2 = 0.67$).

Calculated log-reductions and rates of reduction for *V. parahaemolyticus* at four different flow rates are shown in Table 2.1. The 15 L/min flow rate resulted in the lowest reduction of *V. parahaemolyticus* (2.39 log MPN/g). Increasing the depuration flow rate to 20 L/min or 25 L/min for 5 days enhanced the reduction of *V. parahaemolyticus* to 2.68 log MPN/g and 2.80 log MPN/g, respectively (Table 2.1). Further increase in flow rate to 35 L/min resulted in an average reduction of 3.39 log MPN/g of *V. parahaemolyticus* in oysters after 5 days. As the flow rate increased from 15 L/min to 35 L/min, the rate of *V. parahaemolyticus* reduction

significantly increased from 0.42 to 0.64 log reduction/day ($P < 0.05$). However, none of these depuration treatments achieved FDA's targeted 3.52 (MPN/g) log reduction of *V. parahaemolyticus* for post-harvest shellfish processing (FDA, 2015). This agrees with our previous study which reported that depuration at 12.5 °C in 25 L/min did not achieve > 3.52 log MPN/g reduction (3.33 log MPN/g) (Phuvasate et al., 2012). Multiple depuration studies have demonstrated significant oyster-to-oyster variability which makes validation of the efficacy of a biologically-driven process especially challenging. This result with a > 3.0 log MPN/g mean reduction exhibits the effectiveness of high flow rate depuration. However, a validation of post-harvest process to achieved 3 log reduction for oyster harvested from Pacific Coast proposed by FDA need to confirm consistency (FDA, 2015).

Based on mixed model ANOVA, the difference of mean survivors of *V. parahaemolyticus* is most significantly impacted by duration of depuration (days). Statistically significant difference on mean reduction and rate of reduction of *V. parahaemolyticus* in flow rates between 35 L/min and other flow rates were also observed ($P<0.05$). There was not a significant difference in the *V. parahaemolyticus* reduction rate at 15 L/min, 20 L/min and 25 L/min, although a trend was apparent. This result confirms that higher flow rate of the depuration may help to enhance the efficacy of depuration in decreasing *V. parahaemolyticus* levels in Pacific oysters. Lewis et al. (2010) found depuration can produce >3.52 log CFU/g reduction of *V. vunificus* with higher flow rate (68 L/min) at ambient

temperature, whereas low flow rate even increased by 0.23 log CFU/g (11 L/min) of *V. vulnificus* after 6 days. This finding indicates that higher flow rate achieved better effect but too low flow rate has negative impact on depuration. Continued increased flow rate on this study needs to be further researched.

The depuration process is achieved by holding oysters in clean seawater to purge contaminants from the digestive tract into water (Blogoslawski & Stewart, 1983). It is believed that the activity of pumping water can directly affect elimination of pathogens in shellfish (FDA, 2015). Therefore, the efficacy of depuration largely depends on oyster's physiological (water-pumping) activity. Oyster pumping rate can be influenced by the flow rate and water temperature; however, individual oyster pumping rate may substantially differ under the same environmental conditions (Loosanoff, 1958; Zu Ermgassen et al., 2013). Higher flow rate speeds up the substance exchange, which ensures *V. parahaemolyticus* in the bulk water to be rapidly sterilized, and avoids uptake of *V. parahaemolyticus* by oysters over again. This may partially explain the impact of increasing flow rate on efficacy of depuration.

Increasing the flow rate of artificial seawater for oyster depuration improved the efficacy of the process in reducing *V. parahaemolyticus* populations in artificially inoculate oysters. This study demonstrated that a depuration process at 12.5°C using 35 L/min can achieve >3.0 log MPN/g reductions of *V.*

parahaemolyticus in the Pacific oysters in five days. Further studies are needed to optimize depuration to meet the >3.52 log MPN/g reduction of *V. parahaemolyticus*.

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Table 2.1 Reduction of *V. parahaemolyticus* (log MPN/g) after 5 days of depuration and rate of reduction (log MPN/g/day) of *V. parahaemolyticus* in oysters using various depuration flow rates at 12.5°C.

	Flow Rate (L/min)			
	15 (n=1)	20 (n=1)	25 (n=4)	35 (n=3)
Reduction on day 5 (log MPN/g)	2.39 ± 0.12[a]A	2.68 ± 0.11A	2.80 ± 0.13A	3.39 ± 0.12B
Rate of reduction* (log MPN/g/day)	0.42	0.48	0.54 ± 0.02	0.64 ± 0.05

[a] Values were reported as means of population± standard error. Data with different letters in the same row are significantly different ($P < 0.05$).

*Data are slopes of log reduction of *V. parahaemolyticus* at different flow rates.

[n] Means the number of replicates for each flow rate. Tests with flow rate of 15 L/min and 20 L/min lack replication of tests.

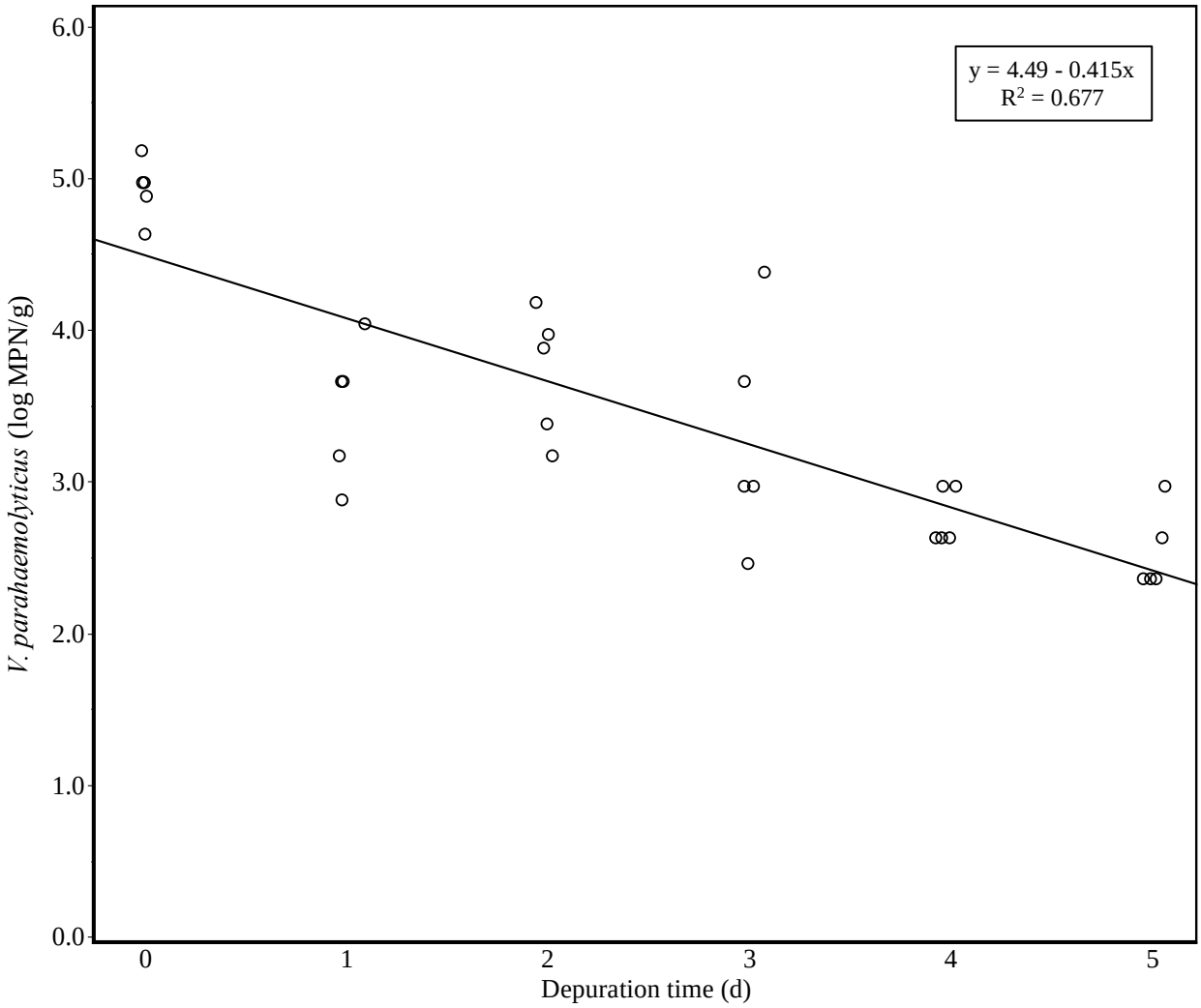

Figure 2.1 Survivors (log MPN/g) of *V. parahaemolyticus* in laboratory-contaminated individual oysters during depuration with flow rate of 15 L/min for up to 5 days.
Markers indicate *V. parahaemolyticus* levels in individual oysters (n = 5 per time point).

Chapter 3 Impact of feeding status on the efficacy of depuration for decontaminating *Vibrio parahaemolyticus* in Pacific Oysters (*Crassostrea gigas*)

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3.1 Abstract

Vibrio parahaemolyticus causes acute human gastroenteritis and is often linked to consumption of raw oysters. Previous investigations indicated that refrigerated seawater depuration at 12.5°C could significantly reduce *V. parahaemolyticus* contamination in Pacific oysters; however, further optimization is necessary to achieve the regulatory target of >3.52 log MPN/g reduction. The current study investigated influences of algal feeding on efficacy of depuration to reduce *V. parahaemolyticus* in raw oysters. A *V. parahaemolyticus* cocktail (10290, 10292, 10293, BE 98-2029, 027-1c1) was mixed in artificial seawater (70 L) to inoculate oysters (n = 35) at 4-5 log MPN/g. Inoculated oysters were subjected to depuration with feed (algae = 0.036 ml/gram of oyster) and without feed at 12.5°C. Oysters (n = 5) were analyzed for *V. parahaemolyticus* using a three-tube most probable number (MPN) method after 0, 1, 3, 5, and 6 days of depuration. Depuration (6 days) achieved average *V. parahaemolyticus* reductions of 2.75 log MPN/g and 3.03 log MPN/g in the fed and unfed systems, respectively; however, feeding status did not significantly impact the efficacy of depuration to reduce *V. parahaemolyticus* in Pacific oysters. Further optimization of depuration is necessary to achieve the regulatory target for *V. parahaemolyticus* decontamination in raw oysters.

3.2 Introduction

Vibrio parahaemolyticus is the leading cause of foodborne illnesses associated with consumption of contaminated seafood, particularly oysters (CDC, 2013). Consumption of raw oysters contaminated with high levels of *V. parahaemolyticus* (10^5CFU/g) may lead to development of acute gastroenteritis (DePaola et al., 1990; Levine et al., 1993). It is estimated that around 4,600 cases of *V. parahaemolyticus* infections occur each year in the U.S. with 62% of them (2,800 cases) associated with consumption of raw oysters (FDA, 2005). FDA (2015) required that post-harvest process must reduce *Vibrio* spp. to non-detectable levels (<30MPN/g) and achieve a 3.52 log reduction.

Several post-harvest processes, including quick frozen (IQF) (Liu et al., 2010), thermal treatment (Andrews et al., 2000; Johnston & Brown, 2002), high pressure processing (HPP) (Calik et al., 2002), and irradiation (Despaigne et al., 2001; Tamplin & Capers, 1992), have been evaluated to reduce *V. parahaemolyticus* in oysters. While these processes are effective at reducing *V. parahaemolyticus* populations, they are unsuitable for markets that demand fresh oysters as these processes also kill the animal. The oyster industry is interested in identifying suitable post-harvest processes that maintain oyster viability while also reducing the risk of *V. parahaemolyticus* contamination.

Oysters feed by filtering large volumes of seawater through their digestive system. In nature, this process serves as a mechanism for accumulation of

pathogens, including *V. parahaemolyticus*, from contaminated water (Goyal & Nelson, 1984; Loosanoff, 1958); however, this natural process can also be used to clear bacteria and viruses from the oyster. Depuration, a controlled process of holding shellfish in recirculated, sterilized (UV light) seawater has been demonstrated to reduce *V. parahaemolyticus* in oysters (Blogoslawski & Stewart, 1983). Phuvasate et al. (2012) reported that depuration at reduced temperatures (7-15°C, 5 days) was shown to reduce *V. parahaemolyticus* by >3.0 log MPN/g in oysters. To achieve the FDA target of a 3.52 log CFU/g reduction for post-harvest process (FDA, 2015), further optimization of depuration is needed.

Oyster activity (i.e., filter feeding) can directly affect elimination of pathogens (FDA, 2015). Previous depuration studies have been conducted using artificial seawater that has not been supplemented with nutrients to support oyster viability (Chae et al., 2009). Feeding oysters may increase the filtering activity of oysters (Haure et al., 2003) and potentially improve the clearance of *V. parahaemolyticus* during depuration. Sobsey et al. (1987) investigated the effect of continuous algae treatment on reducing virus in depurated oyster and found no evident enhancement of depurated efficacy. The objective of this study was to determine whether feeding during depuration would have a positive impact on the reduction of *V. parahaemolyticus* in Pacific oysters.

3.3 Methods and Materials

3.3.1 *Vibrio parahaemolyticus* culture preparation

Five clinical strains of *Vibrio parahaemolyticus* 10290 (serotype O4:K12, *tdh*+, *trh*+), 10292 (serotype O6:K18, *tdh*+, *trh*+), 10293 (serotype O1:K56, *tdh*+, *trh*+), BE 98-2029 (serotype O3:K6, *tdh*+, *trh*-), 1C1-O27 (serotype O5:K15, *tdh*+, *trh*-) were obtained from the FDA Pacific Regional Laboratory Northwest (Bothell, WA, USA). Strains were individually grown in 10 ml tryptic soy broth (Difco, Becton, Dickinson, Sparks, MD, USA) supplemented with 1.5% NaCl (TSB-Salt) at 35-37°C for 16-18 h. Each enriched culture was streaked onto a thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) plate and incubated at 37°C for 18-24 h. One single colony from each TCBS plate was transferred into 10 ml TSB-Salt and incubated at 35-37°C for 4 hr. Five enriched cultures of *V. parahaemolyticus* were then pooled into a 50-mL sterile centrifuge tube and harvested by centrifugation at 3000 x *g* at 5°C for 15 min. Pelleted cells were re-suspended in 2% salt solution to produce a culture suspension of 8-9 log CFU/ml (Su et al., 2010).

3.3.2 Oyster preparation and inoculation

Freshly harvested raw Pacific oysters (small size; ~30 grams/oyster) were collected from a Washington oyster farm (Chetlo Harbor Shellfish, Ilwaco, WA) and transported on ice to Oregon State University's Seafood Laboratory (Astoria, OR). Artificial seawater (ASW) was prepared by dissolving Instant Ocean salt

(Aquarium Systems Inc., Mentor, OH) in deionized water to achieve a salinity of 30 ppt (parts per thousand). Oysters were washed with tap water to remove mud and then placed in a HDPE tank (45 × 30 × 30 cm; Nalgene, Rochester, NY) containing 20 L ASW. Oysters were held under ambient conditions (~20°C) with air continuously pumped into the tank overnight to maintain sufficient oxygen levels (Su et al., 2010). For each experiment, oysters (n = 35) were submerged in aerated ASW (20 L) containing the *V. parahaemolyticus* cocktail at a cell density of ~5 log CFU/ml and held overnight at room temperature.

3.3.3 Depuration with algae treatment

Oysters were depurated in a laboratory-scale system composed of a 15 W Gamma UV sterilizer (Current-USA, Inc., Vista, CA), a water chiller (Delta Star, Aqua Logic, Inc., San Diego, CA, USA), temperature controller (set at 12.5°C; Ranco ETC, Mexico), and a recirculating pump (Pan World Co., Ltd, Japan). The depuration process was conducted with 35 oysters in 70 L of ASW (oyster:water = 1:2) with a flow rate of 25 L/min at 12.5°C. Algae (0.036 ml/g; Shellfish Diet 1800, Reed Mariculture Inc., Campbell, CA) were batch fed to oysters (n = 30) every morning in depurated seawater (60 L) for up to 6 days. The control treatment (unfed) was identical with the exception of algae feeding.

3.3.4 Measurement of algae consumption and oyster gaping activity

Water samples (50 ml) were collected every day at three time points (10 min after daily addition of algae, 10 hrs into feeding, 24 hrs into feeding) for up to 6 days. At these time points, the remaining algae concentration in depurated seawater was determined by visual enumeration using a counting chamber (Hausser Scientific Company, Horsham, PA) and laboratory microscope (Amscope, Inc., Irvine, CA). Absorbance of water (400 nm) was analyzed by spectrophotometer (Shimadzu Corporation, Japan) measure turbidity of water. Rate of algae consumption was calculated as consumed algae cell per unit volume per oyster per hour (cell/ml/oyster/hour).

Movement of oysters was monitored by a Gape-O-Meter (Pacific Shellfish Institute, Olympia, WA) in both fed and unfed groups. The Gape-O-Meter consists of rectangular bars containing an electronic device that measures the distance between the bar and magnetic sensor. Three oysters in each group were glued to each bar with a magnetic sensor attached to the upper shell. The distances were recorded every 5 min for 5 days and the activity of each oyster was expressed as percentage of maximum distance recorded.

3.3.5 Analysis of *V. parahaemolyticus* in oysters

Concentrations of *V. parahaemolyticus* in oysters were determined by using the three-tube most-probable-number (MPN) method (Kaysner & DePaola, 2004).

Oysters (n = 5/time point) were randomly selected for analysis at each time point (i.e., days 0, 1, 3 5). Individual oysters were transferred from the depuration tank, shucked with a sterile shucking knife, and the oyster meat was aseptically transferred to a sterile blender jar. Oyster meat was homogenized with an equal volume of sterile alkaline phosphate buffer saline (PBS; pH 7.4) for 1 min at high speed by a laboratory blender (Waring Laboratory, Torrington, CT). Twenty grams of homogenized oyster sample (1:2 dilution) was mixed with 80 ml of PBS to prepare 1:10 dilution sample suspension. Additional 10-fold dilutions of the sample suspension were prepared using PBS. Sample dilutions were individually inoculated into 3 tubes of alkaline peptone water (APW; pH 8.5; Difco, Becton Dickinson). APW tubes were incubated at 37°C for 16-18 h. 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) plates and incubated at 35-37°C for 18-24 h. Round, green or bluish colonies with 2-3 mm diameter on a TCBS plate after incubation was considered positive for *V. parahaemolyticus*. The efficacy of the UV sterilizer in eliminating *V. parahaemolyticus* in artificial seawater during depuration was verified by three-tube most-probable-number (MPN) method. Fifty milliliter of original seawater sample and 10-fold dilution sample suspensions were analyzed by inoculating in APW tubes. Results were confirmed by TCBS agar as mentioned above.

3.3.6 Statistical analysis

Log-transformed microbiological population levels, rate of algae consumption and valve openness of osyter were statistically analyzed using mixed model, one way Analysis of Variance (ANOVA), and multiple-comparison of Tukey-Kramer using JMP (SAS Institute Inc, Cary, NC). Significant differences between treatment group and control group during depuration were established at $P < 0.05$. Linear fit was used to describe the rate of reduction of *V. parahaemolyticus* over time.

3.4 Results and Discussion

The correlation between algae concentration (cell/ml) in seawater determined by direct microscopic count and absorbance ($OD_{400\ nm}$) is shown in Figure 3.1. Previous studies have utilized spectrophotometric measurement to estimate algal cell density during their growth in monoculture and typically focus on maximum absorbance wavelengths associated with chlorophyll (677-688 nm) (Rodrigues et al., 2011; Santos-Ballardo et al., 2015). The shellfish diet used in the current study is a non-viable mixture of various microalgae (*Isochrysis, Pavlova, Tetraselmis, Chaetoceros calcitrans, Thalossiosira* spp.) that range in size from 4-20 μm (Rikard & Walton, 2012). Spectral absorbance characteristics of individual algae species within a population can express different absorbance ranges even at the same concentration and vary over time and between locations (Knuckey et al., 2005; Lohrenz et al., 1999). Based on the relatively low R^2 value for the

spectrophotometric measurement, direct microscopic counts were used to determine algal cell density for the remainder of the study. A critical aspect of the depuration system is the decontamination of water that contains viable *V. parahaemolyticus* cells that have been purged by the oysters. Water decontamination occurs via UV light as the water is recirculated through the depuration system. Increased turbidity of the water due to algae and the predicted increase in fecal material from feeding may negatively impact the efficacy of sanitation treatment by UV light (FDA, 2015). Seawater samples were collected and tested to verify that the UV treatment was effective in both fed and unfed systems (data not shown).

An example of algae feeding schedule and changes in algal concentrations throughout the depuration treatment of oysters is shown in in Figure 3.2. As expected, algae cell density declined each day as a function of oyster feeding. The slope of the daily algal reduction was used to calculate an average rate of algal consumption of approximately 1400 ± 300 algae cells/ml/oyster/hour throughout the 6-day depuration. Further observations quantifying activity of fed and unfed oysters during depuration were collected to infer oyster pumping activity (Figure 3.3). On average, there is a decreasing trend of openness over time in the fed group. The openness of unfed oysters was significantly lower on day 2 (32.5% openness vs. 56.2% openness for fed oysters); however, the activity of unfed oysters recovered to comparable openness by day 3 and held throughout the remainder of

the depuration treatment. Overall, there was not a significant difference in oyster openness during depuration based on feeding status ($P>0.05$). Higgins (1980) previously reported that continuously fed Eastern oysters showed a higher activity (94.3% valve openness) compared to starved oysters (35.1% openness) for 3 days by holding oysters in aerated seawater; however, variables that influence oyster activity are numerous and interdependent. Previous studies have demonstrated that combinations of feed availability and bulk water flow differentially influence oyster activity (Wilson-Ormond et al., 1997). Valve openness is associated with oxygen and food acquisition, and, as expected, activity related to oxygen intake will continue in the absence of feed (Haure et al., 2003). Results from the current study suggest that valve openness during depuration primary serves to satisfy the oxygen needs of the oyster and that feeding status is a minor contributor to this observation. However, it is possible that modifications could be made to the system (feed quality, timing of feeding, water temperature, water flow rate, etc) that could enhance the differences between oysters depending on feeding status.

The primary objective of this study was to determine whether feeding oysters during depuration would enhance the removal of *V. parahaemolyticus.* A comparison of the efficacy of depuration to reduce *V. parahaemolyticus* in oysters with and without algal feeding is shown in Figure 3.4. Initial *V. parahaemolyticus* concentration in oysters averaged 5.59 log MPN/g (standard error = 0.22). *V. parahaemolyticus* populations were reduced to 3.40 log MPN/g in fed oysters and

to 3.12 log MPN/g in unfed oysters after 5 days of depuration. Overall, depuration resulted in a significant reduction of *V. parahaemolyticus*; however, there was not a significant difference in *V. parahaemolyticus* reduction based on feeding status ($P>0.05$). Previous studies in unfed depuration systems using comparable settings (temperature, flow rate, duration) have reported comparable reductions in *V. parahaemolyticus* in Pacific oysters (Ming et al., 2017; Phuvasate et al., 2012). Sobsey et al. (1987) found that algae treat in depuration water had no appreciable effect on the rates of reduction of virus (poliovirus and Hepatitis A Virus) in Eastern oysters. Neither set of depuration conditions (fed or unfed) were able to achieve the FDA targeted *V. parahaemolyticus* reduction of >3.52 log MPN/g, even with the depuration extended to 6 days (Figure 4.4). Average *V. parahaemolyticus* reduction rates were estimated to be 0.38 log MPN/g/day for fed oysters and 0.42 log MPN/g/day for unfed oysters. These rates predict that depuration treatment would need to extend beyond 8 days to achieve the targeted reduction; however, this extended time would be too costly to the industry and would likely result in reduced viability and quality. However, the predictability of these rates should be cautioned due to the variability of biological activity of individual oysters as evidenced by the relatively low R^2 values (0.65-0.66).

This study has demonstrated that the feeding status of oysters has no significant impact on the efficacy of depuration to reduce *V. parahaemolyticus* levels in Pacific oysters. Further optimization of the depuration process is needed

to achieve the targeted reduction of >3.52 log MPN/g of *V. parahaemolyticus* population in Pacific oysters.

3.5 Acknowledgments

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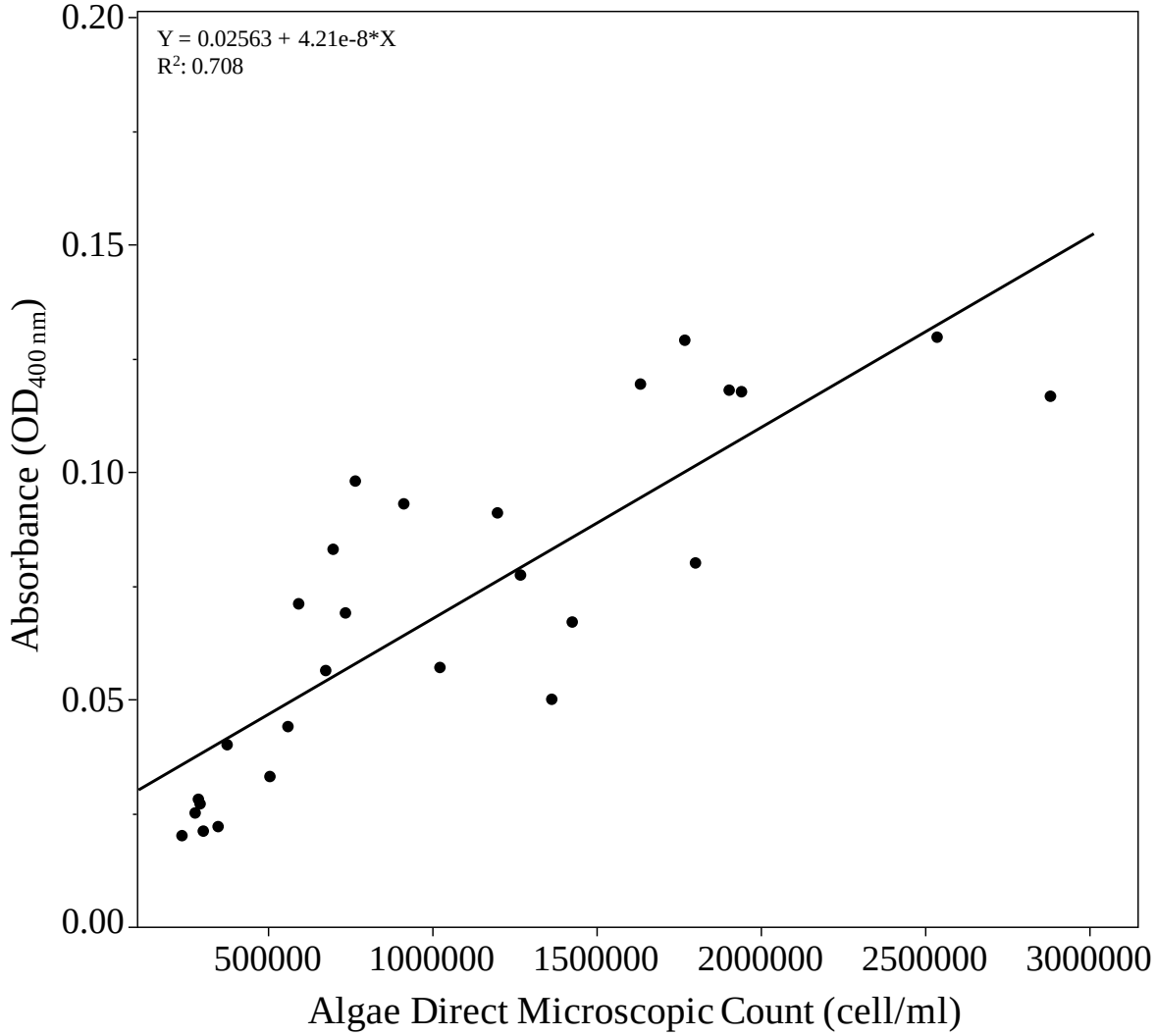

Figure 3.1 Correlation of absorbance ($OD_{400\ nm}$) with algae cell density (cell/ml) as measured by direct microscopic count in artificial seawater (n = 4).

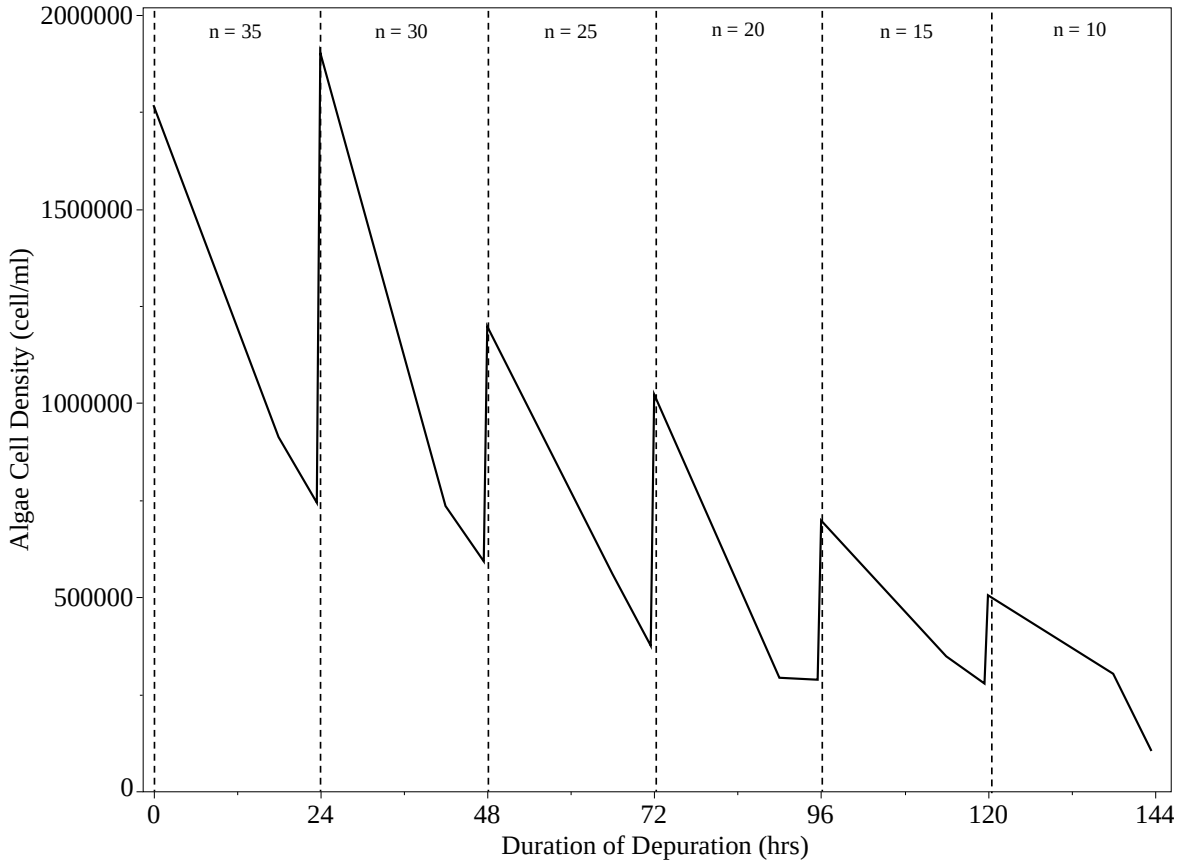

Figure 3.2 Example of changes in algal cell density (cells/ml) due to oyster feeding throughout depuration period (6 days at 12.5°C).
Dashed lines indicate time points of feeding (every 24 hrs). The number of oysters in the depuration tank during each feeding period is displayed at the top of the figure.

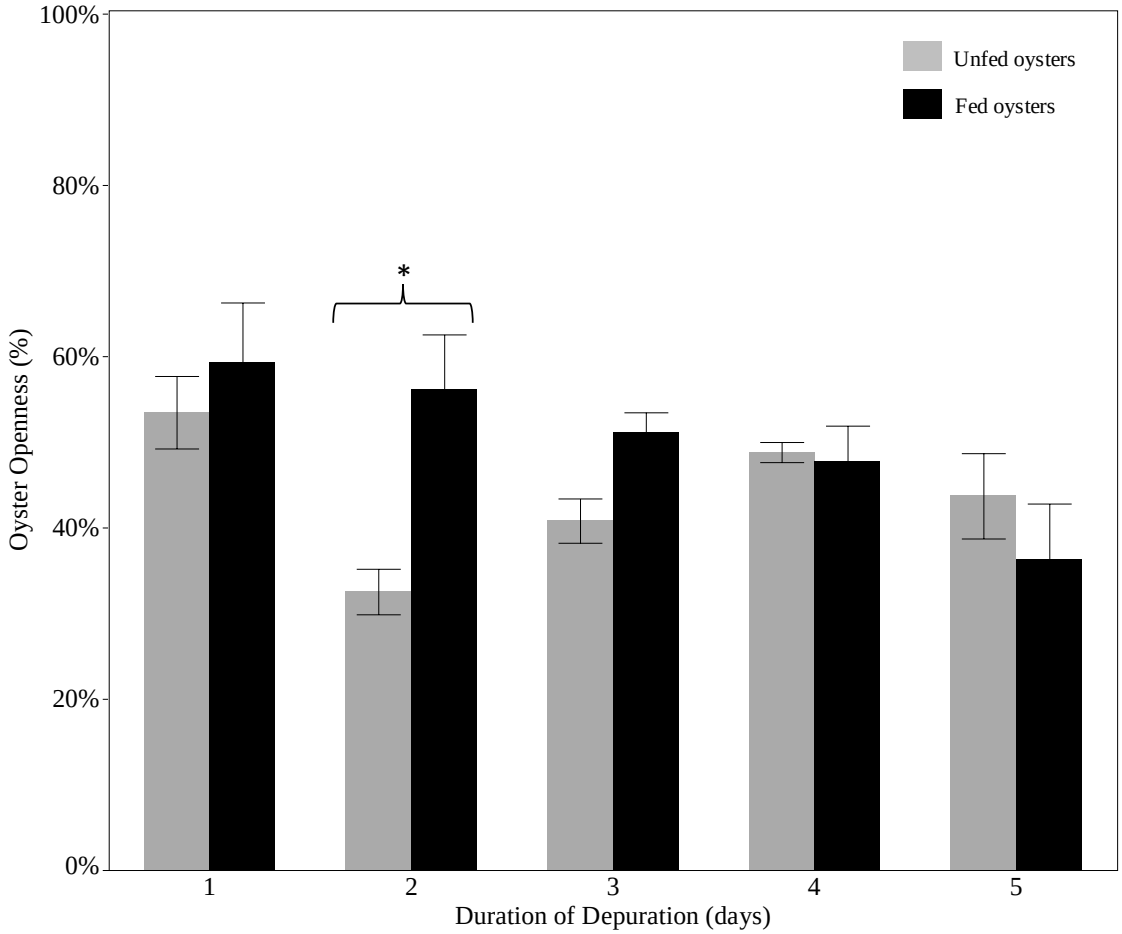

Figure 3.3 Oyster valve openness (% of time) throughout depuration in systems were oysters were fed algae or remained unfed.
Each bar represents the mean openness (n = 3) with bars indicating standard error.
* indicates a significant difference in the openness between fed and unfed oysters ($P<0.05$).

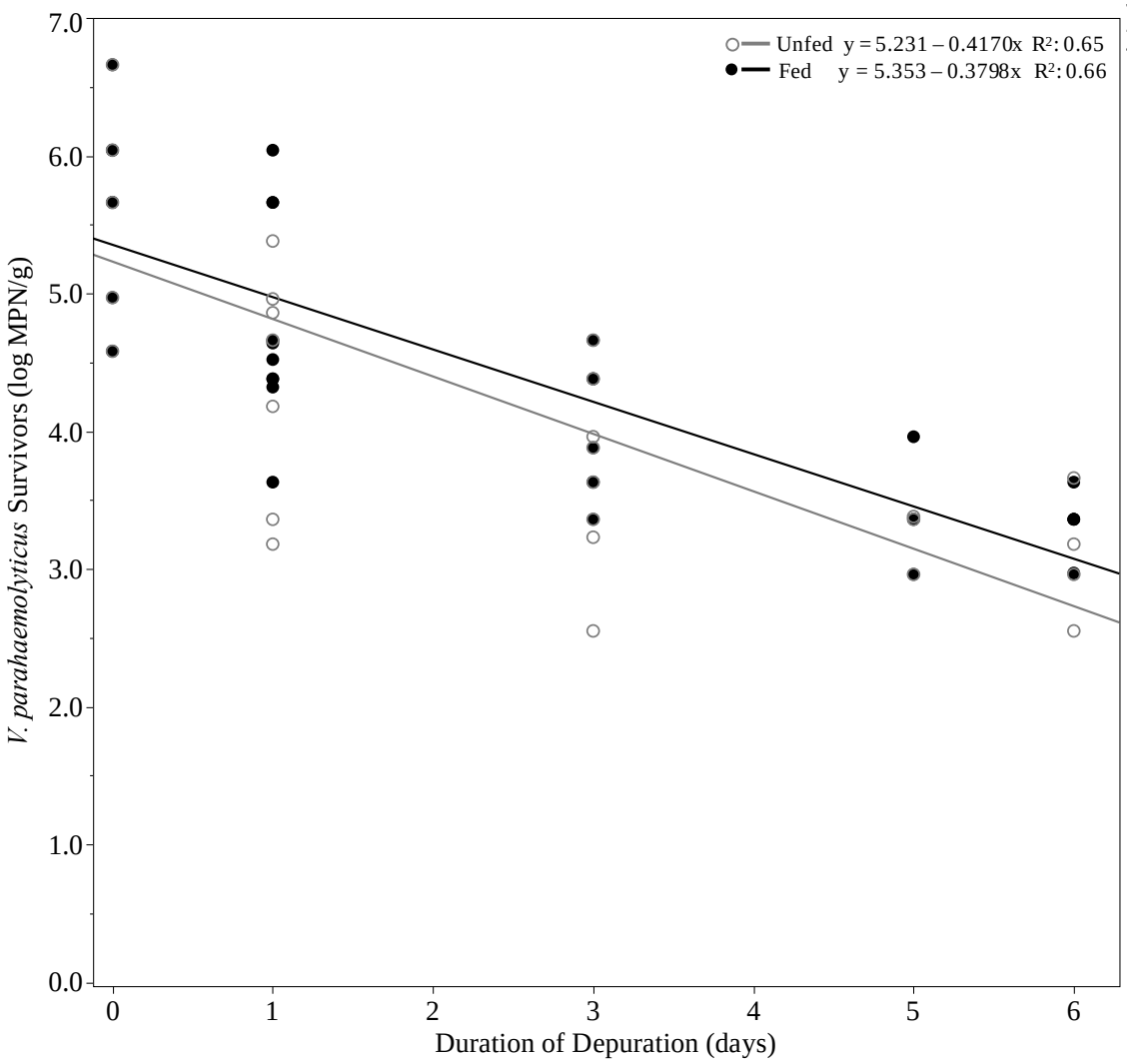

Figure 3.4 Decrease in *Vibrio parahaemolyticus* contamination (log MPN/g) in Pacific oysters (*Crassostrea gigas*) during depuration using flow rate of 25L/min at 12.5°C with and without algal feeding.
Markers indicate *V. parahaemolyticus* cell density in individual oysters.

Chapter 4 General Conclusion

Vibrio parahaemolyticus is one of the leading cause of human gastroenteritis associated with seafood (Letchumanan et al., 2014). This study was aimed to investigate effects of flow rate and feeding treatment on depuration process in reduction of *V. parahaemolyticus* levels in Pacific oysters. Our long-term goal is to optimize this process for adoption by the industry to decrease risks of *V. parahaemolyticus* infections caused by raw oyster consumption.

Studies were conducted to investigate the impact of flow rate in depuration systems to increase its efficacy in reducing *V. parahaemolyticus* (10290, 19292, 10293, BE 98-2029, O27-1c1) populations in Pacific oysters (*Crassostrea gigas*). Depuration process with flow rate of 15 L/min deceased *V. parahaemolyticus* in artificial contaminated oysters by 2.39 log MPN/g after 5 days of in artificial seawater (ASW) at 12.5 ˚C. Greater reductions of *V. parahaemolyticus* populations (2.80 and 3.39 log MPN/g) in oysters were observed through depuration with increased flow rates (25 and 35 L/min), respectively. The highest flow rate in this study (35 L/min) significantly enhanced the ability of depuration process for decontaminating *V. parahaemolyticus* in oysters.

The impact of feeding treatment on the efficacy of depuration in decreasing *V. parahaemolyticus* levels in oysters was studied. A significant difference of valve activity was observed between fed oysters and unfed oysters only on second day of

depuration. Feeding treatment had no significant enhancement in reduction of *V. parahaemolyticus* after five days of depuration at 12.5℃.

In summary, increasing the flow rate of depuration system (to 35 L/min) is capable of increasing the efficacy of depuration to reduce *V. parahaemolyticus* levels in oysters. Feeding status of oysters does not impact the efficacy of depuration. Despite improvements in the efficacy of depuration, we have not found a combination of depuration parameters that achieve FDA's requirement of >3.52 log MPN/g reduction of *V. parahaemolyticus* in Pacific oysters. Future studies are needed to optimize the depuration process for efficient commercial application by the shellfish industry.

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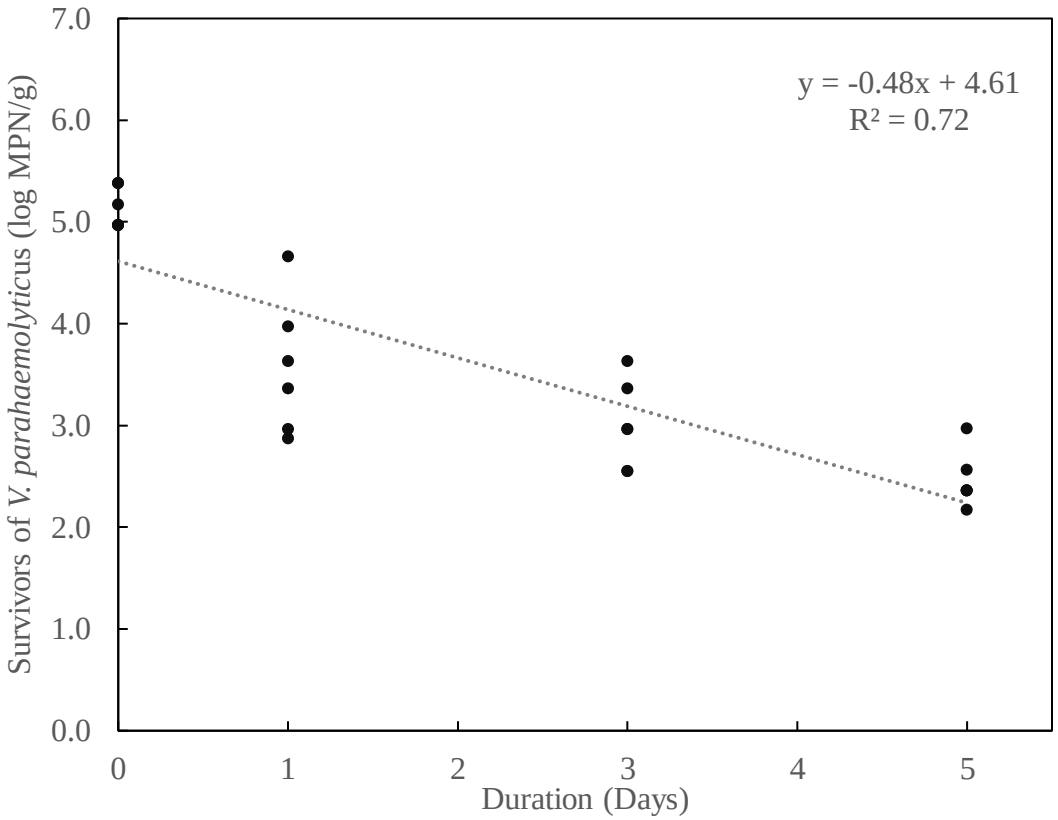
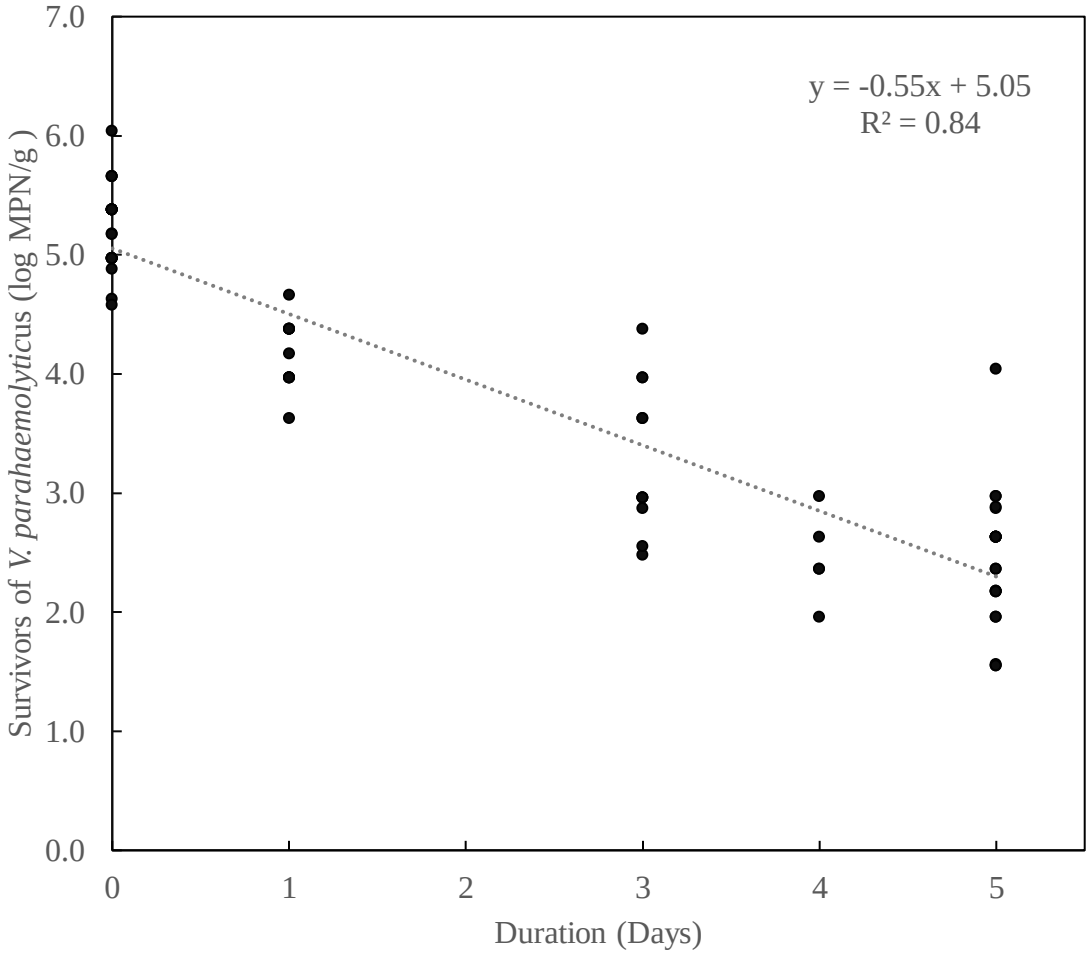
Appendices



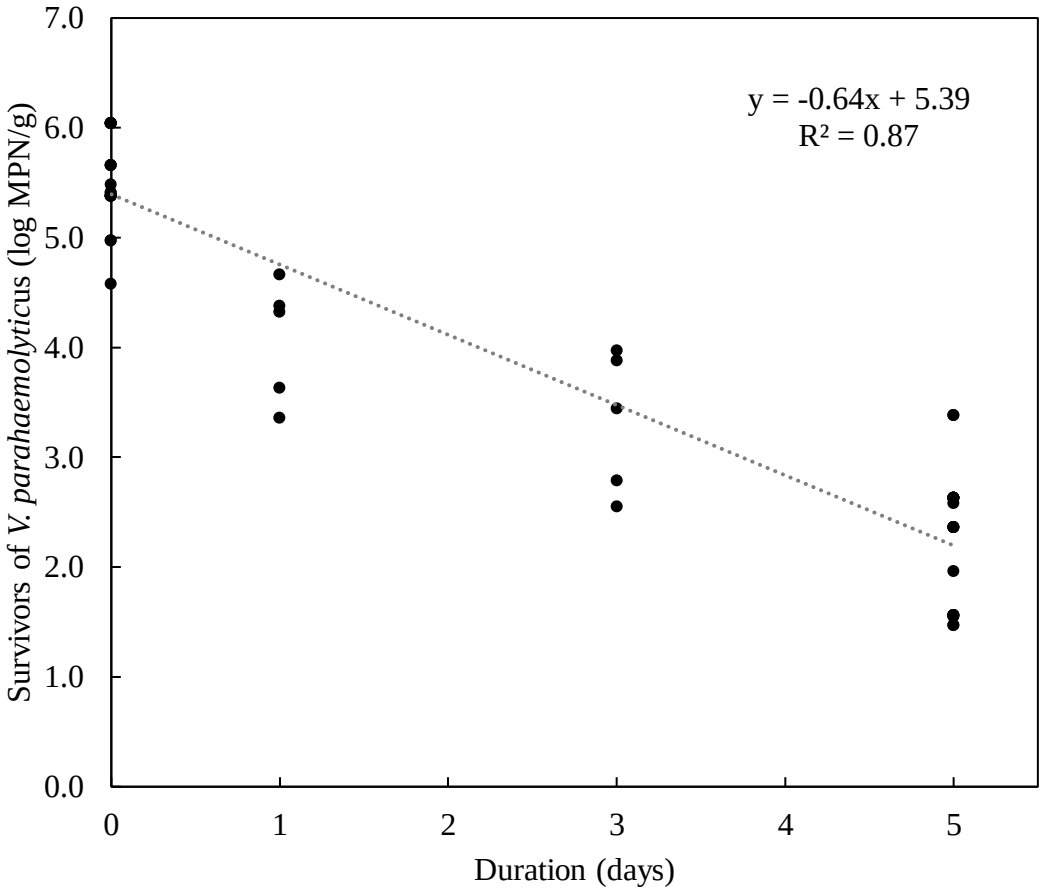
Appendix A. Survivors (log MPN/g) of *V. parahaemolyticus* in laboratory-contaminated individual oysters during depuration with flow rate of 20 L/min for up to 5 days.

Markers indicate *V. parahaemolyticus* levels in individual oysters (n = 5 per time point).



Appendix B. Survivors (log MPN/g) of *V. parahaemolyticus* in laboratory-contaminated individual oysters during depuration with flow rate of 25 L/min for up to 5 days.

Markers indicate *V. parahaemolyticus* levels in individual oysters (n = 5 per time point).



Appendix C. Survivors (log MPN/g) of *V. parahaemolyticus* in laboratory-contaminated individual oysters during depuration with flow rate of 35 L/min for up to 5 days.

Markers indicate *V. parahaemolyticus* levels in individual oysters (n = 5 per time point).