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Abstract approved:

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Oysters are filter-feeding bivalves, which filter water for nutrients and often accumulate contaminants and human pathogens such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* naturally occurring in the marine environment. These naturally occurring pathogens have been frequently isolated from raw shellfish, particularly oyster, in the United States and are recognized as the leading causes of human gastroenteritis associated with seafood consumption. Human illness caused by consumption of raw oyster contaminated with *V. parahaemolyticus* and *Vibrio vulnificus* typically results in reduced sales of oysters and a consequent significant financial burden for the producers.

The United States produces more than 27 million pounds of oysters each year with a large portion of them being produced from the coastal water of the Gulf of Mexico. It is estimated that 20 million Americans eat raw shellfish and consumption of raw oyster

is responsible for about 95% of all deaths associated with seafood consumption in the U.S., making raw oysters one of the most hazardous seafoods. Several post-harvest processes, including low temperature pasteurization, freezing, high pressure processing and irradiation, have been reported capable of reducing *Vibrio* contamination in raw oysters. However, most of them require either a significant amount of initial investment or operation costs, and oysters are often killed during processing. Cost-effective post-harvest processing for reducing *V. parahaemolyticus* in raw oysters without significant adverse effects on the oysters remains to be developed. This study was conducted to determine impacts of low-temperature (15, 10 and 5°C) depuration and frozen storage on reducing *V. parahaemolyticus* and *V. vulnificus* in raw oysters.

Depuration of the Gulf oyster (*Crassostrea virginica*) with electrolyzed oxidizing (EO) water (chlorine, 30 ppm; pH 2.82; oxidation-reduction potential, 1,131mV) containing 3% NaCl was found ineffective on reducing both *V. parahaemolyticus* and *V. vulnificus* in the oysters. Reductions of *V. parahaemolyticus* and *V. vulnificus* in oyster after 48 h of EO water depuration at 22°C were limited to 0.7 and 1.4 log MPN/g, respectively. Depuration with EO water at lower temperatures did not enhance reductions of *Vibrio* in the oysters.

Greater reductions of *V. parahaemolyticus* (1.2 log MPN/g) and *V. vulnificus* (2.0 log MPN/g) were observed when the oysters were depurated with artificial seawater (ASW) at room temperature (22°C) for 48 h. Decreasing temperature of ASW to 15°C for depuration significantly increased the reductions of *V. parahaemolyticus* and *V. vulnificus* to 2.1 and 2.9 log MPN/g, respectively, after 48 h of process. However, depuration of oyster in ASW at 10 and 5°C were found less effective than at 15°C in reducing *Vibrio* in

the Gulf oysters. An extended depuration with ASW at 15°C for 96 h was capable of achieving 2.6 and 3.3 log MPN/g of reductions of *V. parahaemolyticus* and *V. vulnificus*, respectively, in the Gulf oysters.

Study of effects of frozen storage at -10, -23 and -30°C on reducing *V. parahaemolyticus* in raw half-shell Pacific oyster (*Crassostrea gigas*) found that the population of the bacterium decreased faster in oysters stored at -10 than at -23 or -30°C. Holding half-shell Pacific oyster at -10°C for three months or at -23°C for four months was capable of achieving a greater than 3-log (MPN/g) reduction of *V. parahaemolyticus* in the Pacific oyster.

Low-Temperature Post-Harvest Processing for Reducing *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Raw Oysters

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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.

Minjung Chae, Author

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Chapter 1

LITERATURE REVIEW

Members of *Vibrios* are classified as gram-negative, curved and rod-shaped bacteria and are known to be natural inhabitants of the estuarine and marine environments (Table 1.1) (Hollis and others 1976; Wachsmuth and others 1980; Bachman and others 1983; Colwell 1984; Spira 1984). Most of the *Vibrio* species are harmless environmental bacteria that coexist with other estuarine and marine life whereas a few species, such as non-01 *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, are known to be pathogenic to humans (Blake and others 1980; Bachman and others 1983; Desmarchelier 1984). Although pathogenic strains of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* have been recovered from coastal waters of the United States, only *V. parahaemolyticus* and *V. vulnificus* are frequently isolated from a variety of raw seafoods in the U.S. and are recognized as the leading causes of human gastroenteritis associated with seafood (particularly oyster) consumption (Blake and others 1980; Joseph and others1982; Oliver and others 1983; Colwell 1984).

Human illness caused by consumption of raw oysters (and other raw shellfish) contaminated with *V. parahaemolyticus* and/or *V. vulnificus* typically results in reduced sales of oysters, and a consequent significant financial burden for the producers. It is estimated that 20 million Americans eat raw shellfish, and consumption of raw and undercooked shellfish has been reported to account for more than 90 percent of 113,000 cases of seafood poisoning each year in the U.S. (Mead and others 1999). These naturally occurring human pathogens are the major food safety concern for the shellfish industries and consumers.

Table 1.1 Biochemical characteristics of *Vibrio* species commonly encountered in seafood (Data adapted from USFDA 1998)

		V. parahaemolyticus	*V. vulnificus*	*V. cholerae*	*V. algi nolyticus*	*V. mimicus*	*V. fluvialis*	*V. furnissii*	*V. Hollisae*	*V. metschnikovii*
TCBS agar		G [a]	G	Y [b]	Y	G	Y	Y	NG [c]	Y
mCPC agar		NG	Y	P [d]	NG	NG	NG	NG	NG	NG
Oxidase		+	+	+	+	+	+	+	+	-
Gelatinase		+	+	+	+	+	+	+	-	+
Urease		V [e]	-	-	-	-	-	-	-	-
Arginine dihydrolase		-	-	-	-	-	+	+	-	+
Ornithine decarboxylase		+	+	+	+	+	-	-	-	-
Lysine decarboxylase		+	+	+	+	+	-	-	-	+
Acid production from:	Sucrose	-	-	+	+	-	+	+	-	+
	D-Cellobiose	V	+	-	-	-	+	-	-	-
	Lactose	-	+	-	-	-	-	-	-	-
	Arabinose	+	-	-	-	-	+	+	+	-
	D-Mannose	+	+	+	+	+	+	+	+	+
	D-Mannitol	+	V	+	+	+	+	+	-	+
Sensitivity to:	10 µg O/129 [f]	R [g]	S [h]	S	R	S	R	R	ND [i]	S
	150 µg O/129	S	S	S	S	S	S	S	ND	S

[a] G = green [b] Y = yellow [c] NG = no or poor growth [d] P = purple [e] V = variable among strains
[f] O/129 = vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) [g] R =resistant [h] S = susceptible [i] ND= not determined
TCBS: thiosulfate-citrate-bile salts-sucrose; mCPC: modified cellobiose-polymyxin B-colistin; (+): positive; (-): negative

1.1 Epidemiology of *Vibrio parahaemolyticus* and *Vibrio vulnificus*

Shellfish are filter-feeding bivalves, which filter water for nutrients and often accumulate contaminants and bacteria including *Vibrio* spp. from the surrounding water. Although all kinds of shellfish, including oyster, mussel, clam, shrimp, and crab, can be contaminated with *Vibrio* at harvest, a high risk of *Vibrio* infection is usually in association with raw oyster consumption because it is the most abundant shellfish harvested around the world (FAO 1998) and commonly consumed raw. In the U.S., more than 27 million pounds of oysters are harvested each year and most of them are sold live or shucked without further processing (Hardesty 2001). It is estimated that one in 2,000 meals of raw molluscan shellfish serves as the vehicle for *Vibrio* infection (Ahmed 1991) and consumption of raw oyster is responsible for about 95% of all deaths associated with seafood consumption in the U.S. (Oliver 1989), making raw shellfish one of the most hazardous seafoods (Rippey 1994). In 2005, a total of 546 cases of *Vibrio* infections (excluding toxigenic *V. cholerae*) in the U.S were reported to the Cholera and other *Vibrio* Illness Surveillance System of the Centers for Disease Control and Prevention (CDC 2006a). Among them, 232 victims were hospitalized and 40 of them died. Nine *Vibrio* species were isolated from the patients with *V. parahaemolyticus* being the leading cause of infections and *V. vulnificus* being the main cause of death (Table 1.2).

1.1.1 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus can grow in the presence of 1-8% of salt (NaCl) with best growth occurring in the 2-4% range (Sakazaki 1979). Optimal temperatures for *V.*

Table 1.2 *Vibrio* speices (excluding toxigenic *V. cholerae*) isolated from patients suffered from *Vibrio* infections in non-Gulf and Gulf States of the U.S. in 2005 (Data adapted from CDC 2006a)

	Isolates		Patients		Hospitalized		Deaths	
Vibrio Species	Non-Gulf	Gulf	Non-Gulf	Gulf	Non-Gulf	Gulf	non-Gulf	Gulf
V. alginolyticus	40	23	40	23	9	5	0	0
V. cholera (non-toxigenic)	35	23	33	23	14	12	2	3
V. damsela	4	3	4	3	3	0	0	0
V. fluvialis	17	12	17	11	8	7	1	2
V. furnissii	2	0	2	0	2	0	1	0
V. hollisae	4	3	4	3	3	2	0	0
V. mimicus	6	4	6	4	3	2	1	1
V. parahaemolyticus	168	52	168	50	29	17	0	2
V. vulnificus	37	93	36	85	31	70	10	15
Other	10	0	10	0	3	0	0	0
Species not identified	2	9	1	9	0	4	0	0
Multiple species	13	18	6	8	4	4	1	1
Sub-total	338	240	327	219	109	123	16	24
Total	578		546		232		40	

parahaemolyticus to grow are between 30 and 35°C with an upper limit of 45.3°C (Sakazaki 1983). Growth has been observed over the pH range 4.8-11.0 with 7.6-8.6 being optimal. Table 1.3 lists conditions that limit growth of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. In some extreme conditions (such as starvation and temperature stress), *V. parahaemolyticus* could enter a viable but non-culturable (VBNC) state (Jiang and Chai 1996). Under VBNC state, cells *V. parahaemolytics* remain viable, but will not produce visible colonies on growth media.

Although *V. parahaemolyticus* is recognized as a major cause of seafood-borne gastroenteritis, most strains of this species are not pathogenic to human (Joseph and others 1982). Clinical studies revealed a strong association between enterotoxigenic isolates and a β-type hemolytic activity on Wagatsuma agar named the Kanagawa phenomenon (KP). It has been reported that more than 95% of *V. parahaemolyticus* strains isolated from human illness were capable of producing the hemolytic activity on Wagatsuma agar whereas only 1% or less of environmental isolates have been found to exhibit such an activity (Sakazaki and others 1968; Joseph and others 1982). These findings are best explained perhaps by a process of natural selection of KP+ strains in the intestine and better survival of KP- strains in the environment. Sakazaki and others (1974) demonstrated that KP+ *Vibrios* do, in fact, multiply more rapidly than KP-strains in ligated rabbit loops and postulated that selective multiplication of KP+ organisms occurred in the intestine, even if KP- strains were predominant in the ingested food sample. Conversely, KP- strains appear to survive longer in seawater and grow better at 25°C than the KP+ strains while KP+ strains tend to grow better at 37°C and under acid

conditions (Barrow and Miller 1974). However, the mechanisms that both KP- and KP+ strains correspond to environmental changes remain to be investigated.

The hemolysin responsible for KP was named thermostable direct hemolysin (TDH) because it could not be inactivated by heating at 100°C for 15 min (Fukui and others 2005). The TDH has a molecular weight of 42,000 daltons and is composed of two subunit molecules of approximately 21,000 daltons each (Takeda and others 1978). The TDH is cytotoxic and cardiotoxic in experimental animals (Honda and others 1976). When injected into adult mice, the TDH caused intestinal fluid accumulation in suckling mice (Twedt and others 1980) and was capable of penetrating the intestinal epithelium of infant rabbits (Calia and Johnson 1975). In addition, the TDH can cause damage of erythrocyte membrane by acting as a pore-forming toxin that alters ion flux in intestinal cells and leads to a secretory response and diarrhea (Zhang and Austin 2005).

Although epidemiological investigations revealed a strong tie between the Kanagawa phenomenon (KP) and the pathogenicity of *V. parahaemolyticus*, KP-negative strains that did not produce TDH but a TDH-related hemolysin (TRH) had been isolated from outbreak patients (Honda and others 1987, 1988). The TRH was reported to be immunologically similar but physiochemically different to TDH. Unlike TDH, TRH is labile to heat treatment at 60°C for 10 min. The mechanism of TRH in causing human infection seems to be similar to that of TDH. It induces chloride secretion in human colonic epithelial cells and leads to an altered ion flux (Takahashi and others 2000). Therefore, pathogenic strains of *V. parahaemolyticus* are characterized by their ability to produce TDH and/or TRH and both TDH and TRH are recognized as virulence factors of pathogenic *V. parahaemolyticus*.

Table 1.3 Conditions limit growth of *V. cholerae, V. parahaemolyticus* and *V. vulnificus* Growth (Data adapted from USFDA 2001)

Pathogen	Salt Conc. (%)		pH		Temp. (°C)		Oxygen requirement
	min.	Max.	min.	max.	min.	max.	
V. cholerae	0	6	10	6	10	43	Facultative anaerobe*
V. parahaemolyticus	1	8	11	10	5	45.3	Facultative anaerobe
V. vulnificus	0.5	6	10	5	8	43	Facultative anaerobe

* grows either with or without oxygen.

1.1.2 *Vibrio vulnificus*

Vibrio vulnificus can grow in the environments containing 0.5-6% NaCl with an optimal temperatures for growth between 39-40°C (USFDA 1998). This organism can be differentiated from *V. parahaemolyticus* by its lower tolerance to NaCl, ability to ferment lactose, and inability to ferment sucrose (Hollis and others 1976; Morris and Black 1985). *V. vulnificus* can also enter the viable but non-culturable (VBNC) state when temperature dropped below 10°C (Oliver 1995). Under the VBNC state, cells of *V. vulnificus* change from rods to cocci along with a change in membrane fatty acid profile, increased mechanical resistance of cell wall, and reduced amino acid transport (Linder and Oliver 1989; Weichart and kjelleberg 1996; Weichart and others 1997).

Vibrio vulnificus was first recognized as a pathogenic bacterium associated with shellfish consumption in the late 1980. Prior to that time, many state health officials did not monitor this organism or recognize it as a cause of human infection (Andrew 2004). *V. vulnificus* can be divided into two biotypes, which differs in phenotype, serotype and preferred hosts (Tison and others 1982). Strains of biotype 1 are found ubiquitous in the

estuarine environments and are an opportunistic human pathogen. Strains of biotype 2 appear to be related to marine vertebrates infections, especially in cultured eels, which can cause significant economic losses but can also cause wound infections in humans through handling eels.

Vibrio vulnificus are highly invasive and several factors, including an extracellular hemolysin/cytolysin, an elastolytic protease, the presence of a polysaccharide capsule, and an endotoxigenic lipopolysaccharide, have been concerned as possible virulence determinants for the organism.

V. vulnificus can produce a cytotoxin with a molecular weight of about 56 kDa that is toxic to Chinese hamster ovary (CHO) cells and lytic to erythrocytes. It can produce a zinc metalloprotease that can induce a hemorrhagic reaction in skin by digesting type IV collagen, a key structure of the basement membrane (Miyoshi and others 1998). In addition, *V. vulnificus* may exhibit various type of lipopolysaccharide (LPS) and express an extracellular capsular polysaccharide (CPS) on its cell surface. The symptoms of *V. vulnificus* septicemia as well as the inflammatory response seen in patients with wound infections are typical of the endotoxic activity of LPS molecules, suggesting the LPS molecule could be a major virulence factor. In addition, presence of the CPS on cell surfaces of virulent strains of *V. vulnificus* has been reported to be positively correlated with its virulence in the mouse model and is believed to be essential to *V. vulnificus*'s ability to cause human infection (Yoshida and others 1985; Wright and others 1990). Further more, both LPS and CPS are known potent mediators of bacterial septic shock through the induction of host pyrogenic responses (Strom and Paranjpye 2000). Presence of both CPS and LPS on *V. vulnificus* cell surface may produce a

synergistic effect on inducing inflammation, tissue damage, and septicemic shock during systemic *V. vulnificus* infections.

1.2 Symptoms of *Vibrio* infection

Transmissions of *V. parahaemolyticus and V. vulnificus* infections occur primarily through consumption of raw or undercooked shellfish or by exposure of wounds to warm seawater (Blake 1983). The most common clinical presentation of the infection is self-limited gastroenteritis (59%). However, wound infection (34%), primary septicemia (5%), and other symptoms (2%) may also occur (Daniels and others 2000). Persons who are immunocompromised or who have liver disease are at particular high risk for severe *Vibrio* infections and should be warned to avoid consumption of raw or under- cooked shellfish (Hlady and Klontz 1996).

1.2.1 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is usually regarded as a gastrointestinal pathogen though the organism, in rare cases, can also cause wound infection and septicemia (Bonner and others 1983). Clinical manifestations of *V. parahaemolyticus* infection have included diarrhea (98%), abdominal cramps (82%), nausea (71%), vomiting (52%), headache (42%), fever (27%) and chills (24%) with incubation periods ranging from 4 to 96 h (Morris and others 1985). The illness caused by *V. parahaemolyticus* is often self-limited with a median duration of three days and the majority of infected persons are able to continue their duties to some degrees (Joseph and others 1982). However, in markedly severe cases the infection may cause septicemia that is life-threatening to people having

underlying medical conditions such as liver disease or immune disorders. Two deaths were reported among three cases of wound infections caused by *V. parahaemolyticus* in Louisiana and Mississippi after Hurricane Katrina in 2005 (CDC 2005a).

1.2.2 *Vibrio vulnificus*

Vibrio vulnificus infection has been linked to two distinct syndromes: primary septicemia and wound infection (Blake and other 1979; Tacket and others 1984). Other symptoms associated with the infection include fever (94%), chills (91%), nausea (58%) and hypotension (systolic pressure <85mm; 43%) (Janda and others 1988; Oliver 1989). In some cases, the infected patients will develop secondary lesions, typically at the extremities, which often require surgical amputation (Oliver 1989). One notable clinical finding of this disease is the absence of a significant diarrhea in a large percentage of patients before their septicemic illness.

The development of primary septicemia in a *V. vulnificus* infected patient generally occurs within 24 hours of ingestion of raw seafood containing the organism with an elevated level of iron being observed in the serum in those who have preexisting liver disease (Janda and others 1988). The fatality rate resulting from the development of septicemia is usually high (>50%) and greater than 90% of the suffering patients could become hypotensive within 12 h of being admitted to hospital (Yamamoto and others 1990).

It is interesting that the infection of *V. vulnificus* occurs more frequently in males with 82% of the cases reviewed were males with an average age of 50 years or older (Oliver 1989). It is speculated, though not clear yet, that the infection could be sex-

dependent. Research has shown that male rats had a significant higher mortality rate than female rats when injected intravenously with LPS secreted by *V. vulnificus*.

Patients with *V. vulnificus* wound infection are usually caused by exposure to seawater. Wound infection caused by *V. vulnificus* generally leads to development of symptoms ranging from relatively mild to severe and rapid progressive cellulites and myositis (Blake and other 1979; Kelly and McCormick 1981; Morris and Black 1985). The mortality rate following wound infection is approximately 25% (Oliver 1989). Three deaths were reported among residents of Louisiana and Mississippi States after Hurricane Katrina in 2005 as a result of wound-associated *V. vulnificus* illness (14 cases) (CDC 2005a).

1.3 Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus*

Members of *Vibrio* have been isolated from virtually every geographic area of the U.S. coastal water with a higher frequency reported in states lying along the southern Atlantic coastline and the Gulf Coast (Janda and others 1988). The distribution of *Vibrio* in the marine environment is mainly dependant on the temperature and salinity of seawater (Kelly and McCormick 1981; Singleton and others 1982). Higher numbers of *Vibrio* cells are generally observed in seawaters with temperatures ranging from 17 to 35°C with salinity ranging from 5 to 25 parts per thousand (‰) (Joseph and others 1982; Bonner and others 1983; Morris and Black 1985). Besides temperature and salinity, occurrence of *Vibrio* spp. in seawater can also be influenced by interactions with plankton and the environment. A study of the Rhode River area of the Chesapeake Bay reported that *V. parahaemolyticus* survived in sediment during the winter and were later

released into the water column as temperatures increased, where they became associated with the zooplankton from April to early June (Kaneko and Colwell 1973, 1978). In general, shellfish-borne *Vibrio* infection tends to occur at a higher rate in coastal area in the summer and fall when water is warmer and *Vibrio* counts are higher, which leads to a seasonal occurrence of clinical cases and fatalities traced to the consumption of contaminated seafood (Levine and Griffin 1993).

The ecological study of *V. parahaemolyticus* in Rhode River area of the Chesapeake Bay in Maryland reported that the population of *V. parahaemolyticus* in water increased to 100-10,000 cells/100mL in August when water temperature rose to 25~30°C and decreased to ca. 100 cells/100mL when water temperatures dropped to 6°C in mid-winter (Kaneko and Colwell 1973). A survey conducted between 1984 and 1985 in Nine Coastal States found a seasonal and geographical distribution of *V. parahaemolyticus* with the density of *V. parahaemolyticus* (68 cells/100ml) in seawater was higher in spring (temperature 25°C) than that in winter (temperature 10°C) and the density was higher (44 cell/100ml) in the seawater sampled from the Gulf Coast than from the Pacific Coast (2 cells/100ml) (DePaola and others 1990). A recent study of *V. parahaemolyticus* in Oregon estuaries (Yaquina and Tillamook Bays) between 2002 to 2003 found that densities of *V. parahaemolyticus* in both bays were positively correlated with to water temperature, with higher densities in samples being detected in summer, especially July and August (Duan and Su 2005).

Vibrio vulnificus has been isolated from waters with temperatures ranging from 13 to 31°C with salinities ranging from 0.8 to 34 ‰ (Kelly 1982; Oliver 1982; Oliver and others 1983; Kaysner and others 1987; Kelly and Dan Stroh 1988). Despite its apparent

tolerance of wide ranges of salinity and temperature, *V. vulnificus* is more frequently found in higher densities in water with temperatures of 17 to 31°C and salinities of 15 to 25 ‰ (O'Neill and others 1992). Studies of seawater and oysters collected from Galveston Bay, Texas overlying oyster beds in July (water temperature, 28°C) yielded an average of 2,000 CFU/ml of *V. vulnificus* in the water with approximately 70% of 54 oysters tested were positive for *V. vulnificus* (Kelly and Dizuzzo 1985). No *V. vulnificus* was detected in either seawater or oysters collected from the same area in March when water temperature was about 15°C.

A study examined the seasonal distribution of *V. vulnificus* in Northern Gulf and Atlantic Coast Oysters from July 1994 through September 1995 found that oysters in all Gulf Coast exhibited a similar seasonal distribution of *V. vulnificus* with a consistent large number (average 2,300 MPN/g) detected in the oyster from May through October followed by a gradual reduction to <10 MPN/g in November and December (Motes and others 1998). The density of *V. vulnificus* then increased sharply beginning in late March and reached its highest level in the summer months.

A similar study monitoring the occurrence of *V. vulnificus* in *V. vulnificus* in oysters from reefs in south Louisiana's Plaquemines Parish also reported low levels (3 or 4 to 100 MPN/g) of the organism in oyster meat in the winter months (late December, January and February) with no *V. vulnificus* being detectable in seawater (Andrew and DeBlanc 2002). The density of *V. vulnificus* in the oyster began to rise in late March and continue to climb to a high number (10^4 MPN/g of oyster meat) from June through late September. Although low levels of *V vulnificus* are normally expected in the Gulf oysters,

illnesses and deaths resulted from *V. vulnificus* infection have been reported in nearly every month.

1.4 Incidence of *Vibrio parahaemolyticus* infection

Vibrio parahaemolyticus was first identified as a cause of foodborne illness in Japan in 1950 (Fujino and others 1953). The outbreak involving 272 victims and 20 deaths was traced to the consumption of a boiled and semi-dried sardine preparation. Since the, more than 70% of the food poisoning cases reported in Japan were identified due to ingestion of seafood contaminated with *V. parahaemolyticus* (Sakazaki 1967). In the U.S., the first *V. parahaemolyticus* outbreak occurred in Maryland in 1971 (Molenda and others 1972). A total of 425 persons became ill after consumption of improperly cooked crabs. Subsequently, *V. parahaemolyticus* was recognized as the most common cause of human gastroenteritis associated with seafood consumption. Four major outbreaks of *V. parahaemolyticus* infections involving more than 700 cases of illness associated with raw oyster consumption occurred between 1997 and 1998 in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions of the U.S. (CDC1998, 1999). Among them, the outbreak occurred in Texas in 1998, which was linked to the consumption of raw oysters from Galveston Bay of Texas, was the largest *V. parahaemolyticus* outbreak (416 cases) ever reported in the U.S. (Daniels and others 2000).

Although efforts were made after those outbreaks including an establishment of time-to-temperature regulations limiting the maximum time of exposure of oysters to ambient temperatures post harvest to prevent rapid growth of *V. parahaemolyticus* in

contaminated oysters in the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish (NSSP 2003), incidence of *V. parahaemolyticus* infection continued to be reported in association with raw oyster consumption in the U.S. The organism sickened 14 passengers on board a cruise ship in Alaska after eating raw Alaskan oysters in the summer of 2004 (McLaughlin and others 2005). In the summer of 2006, a *V. parahaemolyticus* outbreak of 177 cases reported in New York City, New York State, Oregon and Washington was linked to contaminated oysters harvested in Washington and British Columbia (CDC 2006b). The number of confirmed cases (72) for this outbreak is greater than the annual average (16) confirmed cases in these areas and the average number reported in the entire U.S. between May and July from 2000 to 2004. The occurrence of these outbreaks indicates that contamination of *V. parahaemolyticus* in oysters remains a food safety concern in the U.S.

1.5 Incidence of *Vibrio vulnificus* infection

The infection appears to be a concern associated with consumption raw oyster from the Gulf Coast. Oysters produced in the Pacific Northwest are generally not considered a vehicle for *V. vulnificus* infection because the water temperature is usually not high enough to allow *V. vulnificus* to multiply to a high density.

The first case of disease caused by *V. vulnificus* was documented in late 1970's (Blake and others 1979). Between 1975 and 1989, there were 115 cases of shellfish-associated *V. vulnificus* infections reported in the U.S. with over 80% of the cases occurred in the southern Gulf areas (Rippey 1992). This pathogen is responsible for an averaged 15 cases of primary septicemia related to shellfish consumption reported in the

U.S. each year between 1989 and 1994 with a mortality rate averaging 45% (Creasy and Glatzer 1995). Most of the cases (85%) occurred between May and October, and only oysters harvested from Gulf Coast States have been implicated (Klontz and others 1988; Levin and Griffin 1993; Rippey 1994). In 1996, *V. vulnificus* caused an outbreak of 16 cases with 3 deaths in Los Angeles in association with the consumption of raw oysters produced from Galveston Bay in Texas and Eloi Bay in Louisiana (CDC 1996). The U.S. Food and Drug Administration reported that *V. vulnificus* caused 30 illnesses with 18 deaths in 2000, 35 illnesses with 17 deaths in 2001, and 21 cases with 12 deaths in 2002 (Glatzer 2002). While the average number of cases reported in an outbreak for *V. vulnificus* infection is usually smaller than that reported for *V. parahaemolyticus* infection, the high mortality rate resulted from *V. vulnificus* infection makes *V. vulnificus* a very dangerous pathogen for the health concern.

1.6 Strategies for preventing *Vibrio parahaemolyticus* and *Vibrio vulnificus* foodborne infection

Polluted water has a long history of serving as a vehicle for *V. cholerae* infection in human and monitoring water quality has been a key strategy used by state health regulators to prevent illnesses caused by *Vibrio* spp. (Andrews 2004). Today, monitoring water quality is still the first line of defense in preventing illness from bacterial pathogens associated with shellfish. However, this line of defense appears to be not enough in preventing foodborne *V. parahaemolyticus* and *V. vulnificus* infections as outbreaks of *V. parahaemolyticus* and *V. vulnificus* infections associated with shellfish consumption

continue to be reported in the U.S. Other strategies including guidelines, education, natural harvesting control, and post harvest treatments (PHT) have been explored.

1.6.1 Guidelines

Following the outbreaks of *V. parahaemolyticus* infection occurred in 1997 and 1998, the U.S. Food and Drug Administration (FDA) provided guidance and recommendations to Interstate Shellfish Sanitation Conference (ISSC) for monitoring *V. parahaemolyticus*, which limit viable *V. parahaemolyticus* to 10,000 or fewer cells per gram of seafood (ISSC 1997). However, examination of shellfish samples by state and federal authorities following the 1998 outbreaks found that overall levels of *V. parahaemolyticus* in most oysters from implicated growing areas were less than 1,000 *Vibrio* cells per gram with some of them as low as 100 cells per gram (Kaysner and DePaola 2000). These findings suggest a possible low infectious dose of the pathogenic strains involved in the outbreaks and that the FDA guidance may not be sufficient to protect consumers from *V. parahaemolyticus* infection associated with raw oyster consumption.

To limit growth of *V. parahaemolyticus* in contaminated oysters, the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish established time-to-temperature regulations that limit the maximum time of exposure of oysters to ambient temperatures. Shellfish harvested for raw consumption need to be cooled down to 10°C (50°F) within 10, 12, and 36 h of harvest when the average monthly maximum air temperature is ≥27°C (81°F), between 19 and 27°C (66-80°F), and <18°C (66°F), respectively (NSSP 2003). In an attempt to reduce *V. parahaemolyticus* infection

associated with oyster consumption, harvest of oysters in Mississippi for raw consumption is limited from mid-September through April. Oyster beds are closed during the warmer summer months and at other times when heavy rains cause an influx of potentially contaminated water. These harvesting practices minimized the incidence of *V. parahaemolyticus* illness from consumption of Mississippi oysters (Andrews 2004).

In addition to temperature controls, harvest practices (such as intertidal harvesting) can also influence levels of *V. parahaemolyticus* in oysters. Oysters are exposed to ambient air before being harvested in the intertidal harvest, which allows *V. parahaemolyticus* to multiply rapidly in oysters especially on warm and sunny days. Nordstrom and others (2004) investigated effect of low-tide exposure of oysters to ambient conditions on *V. parahaemolyticus* levels in oysters and found that the mean densities of *V. parahaemolyticus* in oysters increased to four to eight times of the initial level after exposure to the ambient temperature. The study demonstrated that the densities of *V. parahaemolyticus* could increase in oysters during the low-tide exposure in summer and suggested a modification of the harvest practice, such as avoiding harvest of oysters after intertidal exposure to ambient conditions, could potentially reduce the incidence of *V. parahaemolyticus* infection associated with raw oyster consumption.

1.6.2 Education

The first step in consumer education regarding potential health risks associated with shellfish consumption was to attach a warning sign to the shellfish for sale, especially for those intended for raw consumption (Andrews 2004). The Interstate Shellfish Sanitation Conference (ISSC), an organization formed in 1982 to foster and

promote shellfish sanitation through the cooperation of state and federal control agencies, the shellfish industry, and the academic community has been proactive in developing a variety of educational materials, including consumer bulletins, press releases for newspapers and television, and pamphlets for health care workers (ISSC 2001). In addition, government health authorities have conducted many workshops and lectures for consumers and health care providers aiming at reducing *Vibrio* infections. Unfortunately, these educational strategies have little impact on reducing the number of illnesses and deaths associated with *V. parahaemolyticus* and *V. vulnificus* infections over the past decade. Recently, the U.S. Centers for Disease Control and Prevention reported a 78% increase in incidence of *Vibrio* (mainly *V. parahaemolyticus* and *V. vulnificus*) infections in 2006 from the 1996-1998 baseline (CDC 2007).

1.6.3 Post-harvest treatments (PHT)

Refrigeration has been a most commonly used post-harvest process for preserving quality and extending shelf life of raw oysters. However, recent development in technology has allowed use of high pressure processing, irradiation, low temperature pasteurization, freezing, and chemical processing to inactivate *Vibrio* cells in contaminated oyster. The ISSC has established a standard for developing effective post-harvest treatments, which are capable of reducing *Vibrio* cells in shellfish by a 5-log scale with no *Vibrio* cells being detectable (<3 MPN/g for *V. vulnificus* and <10 CFU/g for *V. parahaemolyticus*) in the oyster after the process (NSSP 2003).

1.6.3.1 High pressure processing

High-hydrostatic pressure processing (HPP) has attracted increased attention as a means of non-thermal treatment of raw or fresh foods for reduction of bacterial load without causing significant changes in appearance, flavor, texture, and nutritional qualities (Styles and others 1991; Berlin and others 1999). The application of HPP on food offers advantages over thermal processing in that microorganisms and detrimental enzymes can be inactivated at ambient or low temperatures without the breaking of covalent bonds which are essential to virtually all flavor, color, and nutritional constituents within a food system (Hoover and others 1989).

Several studies have reported the effectiveness of HPP on inactivating *V. parahaemolyticus* and *V. vulnificus*. Berlin and others (1999) reported that strains (two each) of *V. vulnificus, V. parahaemolyticus* and *V. cholerae* were susceptible to HPP treatment at pressure levels between 200 and 300 MPa. When homogenized raw oysters were inoculated with *V. vulnificus* or *V. parahaemolyticus* to a density of 10^7 CFU/g and subjected to pressure treatment, the bacteria in the oyster homogenate were totally inactivated after a treatment of 200 MPa for 600 s at 25°C.

Calik and others (2002) found that treatments of 345 MPa for 30 and 90 s at 22°C were optimum conditions for reducing *V. parahaemolyticus* in pure culture (7.6×10^6 - 5.5×10^8 CFU/ml) and in oysters (8.4×10^5 - 3.4×10^7 CFU/g), respectively, to non-detectable levels (<10 CFU/mL or CFU/g). Cook (2003) reported that a HPP treatment of 300 MPa for 180 s was sufficient to achieve a >5-log reduction of *V. parahaemolyticus*, including *V. parahaemolyticus* O3:K6 strains, in oysters. A less intense treatment of 250

MPa for 120 s was capable of achieving the same degree (>5-log) of reduction of *V. vulnificus* in oysters.

An added benefit of using HPP for inactivating *Vibrio* spp. in oyster is that the process assists in oyster shucking by destroying the adduct muscle and the shell of oyster will "pop-open" during the process. To prevent the lost of oyster juice during a HPP process, the shell must be held shut by banding or wrapping (Andrews 2004). He and others (2002) reported that HPP treatments of 240 to 275 MPa were optimum for shucking Pacific oyster with minimum changes in appearance. While the HPP could be used for effective reduction of *V. parahaemolyticus* and *V. vulnificus* in oysters, the high costs of initial investment of the high-pressure system makes it not economically feasible to most oyster producers.

1.6.3.2 Irradiation

Irradiation is a non-thermal process capable of destroying bacterial pathogens in foods. Irradiation of molluscan shellfish has been shown could effectively eliminate even the most resistant *Vibrio* pathogens (Kilgen and others 1998; Andrews and others 2002). Study has shown that strain of *V. parahaemolyticus* O3:K6, the most processing resistant strain of pathogenic *Vibrio*, was effectively eliminated with ≤1.5KGy, even when present in large numbers. Andrews and others (2003b) reported that naturally incurred *V. vulnificus* (3-log/g) in oysters was reduced to non-detectable levels with a Cobalt-60 gamma radiation treatment at 0.75 kGy. The irradiated oysters did not develop significant changes in sensory characteristics and maintained a good quality for a shelf-life of >15 days. It has been reported that oyster could survive a irradiation process of low dosages.

Jakabi and others (2003) reported that an irradiation process of <3 kGy did not kill oysters or affect the sensory quality of oysters. Despite of the effectiveness of irradiation process for destroying *V. parahaemolyticus* and *V. vulnificus* in oyster, the needs to handle radioactive materials limit its usage.

1.6.3.3 Heating

The National Shellfish Sanitation Program permits shellfish dealers to use a heat-shock process to prepare shellstock oyster for sucking (NSSP 2003). This process was originally developed to facilitate the sucking process by relaxing the oyster's adductor muscle. Hesselman and others (1999) reported a heat-shock process by holding oysters in 67°C water for 1 to 4 min could significantly reduce *V. vulnificus* and total bacterial levels by 1- to 4-log units in shucked oyster meat (Hesselman and others 1999). Andrews and others (2000) also showed that the use of low temperature pasteurization, by placing the oysters in 55°C water to achieve an internal temperature of 48-50°C for 5 min, was very effective in reducing the pathogens *V. vulnificus* and *V. parahaemolyticus* from 10^5 MPN/g to non-detectable levels. However, a longer processing time of at least 22 min at 52°C was required to totally eliminate 10^6 CFU/g of *V. parahaemolyticus* O3:K6 inoculated to oyster (Andrews and others 2003a).

1.6.3.4 Cooling

Low-temperature storage of oyster has been reported a very effective means in controlling growth of *V. vulnificus* and *V. parahaemolyticus* in oyster post harvest. Some studies suggested that a low-temperature storage could, in fact, result in decreased counts

of both *V. vulnificus* and *V. parahaemolyticus* in oyster during the storage. Cook and Ruple (1989) reported that the multiplication of fecal coliforms and *Vibrios* in oysters harvested from the Gulf Coast was prevented when the oyster was stored at 10°C. Cook (1994) found that multiplication of *V. vulnificus* was retarded for 30 h when oysters were kept at 13°C or below, whereas the bacterial number increased significantly when oysters were held at 18°C or higher.

Storage of shellstock oysters at ≤ 4°C has been capable of reducing *Vibrio* in oysters gradually over time. Kasper and Tamplin (1993) found that the densities of *V. vulnificus* in shellstock oysters stored at 0, 2 and 4°C were all reduced by 1-log unit after 3 days of storage. The population of *V. vulnificus* in the oysters stored at 2 and 4°C remained at the same level through 14 days of storage. However, a 2.5-log reduction of *V. vulnificus* in the oyster was reported after 10 days of storage at 0°C. Thompson and Vanderzant (1976) reported that populations of *V. parahaemolyticus* in shucked oysters decreased from > 11,000 to 0.36 MPN/g after 7 days of storage at 3°C. Muntada-Garriga and others (1995) reported that viable cells of *V. parahaemolyticus* (10^{5-7} CFU/g) in oyster homogenates were completely inactivated by freezing at -18 and -24°C for 15 to 28 weeks depending on initial populations of the microorganism and freezing temperatures.

1.6.3.5 Chemical process

Chemicals used to reduce *Vibrio* numbers in oysters have included the effect of acetic acid in hot sauces and marinades and natural citric acid in lemon juice. These acid products are commonly consumed with raw half-shell oysters (Andrews 2004). Half-shell

oyster treated with 100% lemon juice, 5% citric acid, 10% citric acid or vinegar for 30 min resulted in 2-4 log reduction in the number of *V. vulnificus* (Borazjani and others 2003).

1.6.4 Natural harvest controls

In an attempt to reduce the numbers of *Vibrios* in oysters prior to marketing, several strategies relating to harvesting practices have been proposed and implemented. In Mississippi, oysters intend for raw half shell consumption are allowed to harvest only from mid-September through April. The oyster beds are closed during the warmer summer months and at other times of the year when high river water or heavy rains cause an influx of potentially contaminated water. Since the implementation, no illnesses have been reported to occur from consumption of Mississippi oysters. This may be a result of the harvesting practices or a possible coincidence to the low overall percentage of Gulf oysters from Mississippi that had been sold for half shell consumption (Andrew 2004).

The FDA and ISSC have established time-to-temperature regulations that limit the time oysters are held at ambient temperatures prior to refrigeration (NSSP 2003). However, rapid increase of *V. parahaemolyticus and V. vulnificus* in oyster can occur if oyster are left on board upon harvest and exposed to ambient summer temperature for a certain period (Cook 1997). On-board refrigeration of oyster upon harvest may need to be enforced to prevent rapid increase of *V. parahaemolyticus and V. vulnificus* in oyster.

1.6.4.1 Relaying

Relaying is a process by transferring shellfish before harvest from polluted areas to an unpolluted waterway for natural biological purification. However, increased pollution along coastal line due to introduction of animal waste from farmland into marine environment has resulted in reduced clean area for growing shellfish. Although study has shown that the population of *V. vulnificus* in oyster could be reduced from 3 - 4 log MPN/g to < 10 MPN/g within 7-17 days (Motes and DePaola 1996), the lack of clean and unpolluted marine environment for growing shellfish creates a big challenge for the relaying practice (Roderick and Schneider 1994).

1.6.4.2 Depuration

Depuration is controlled purification process allowing shellfish to purge sand and grit from the gut into tanks of clean seawater (Richards 1988). The process usually leads to a reduction of microbial contaminants in shellfish and therefore increases shelf life of refrigerated products (Fleet 1978). However, studies have shown that depuration with clean seawater was not effective in reducing certain persistent bacteria including *Vibrio* spp. in shellfish because of the colonization of those bacteria in the intestinal tracts. Eyles and Davey (1984) found no significant difference in mean counts of naturally occurring *V. parahaemolyticus* between depurated and non-depurated oysters. Kelly and Dinuzzo (1985) reported that oysters required 16 days to depurate laboratory-contaminated *V. vulnificus* to non-detectable levels. Tamplin and Capers (1992) found that depuration with temperatures greater than 23C° caused *V. vulnificus* numbers to increase in the oysters.

1.7 Factors affecting depuration

1.7.1 Water temperature

The efficacy of depuration for reducing bacterial loads in oyster is related to the water-pumping activity of oyster, which can vary at different temperatures (Rowse and Fleet 1984). Each shellfish species will pump at different ranges of temperatures and shellfish grown at cold water tend to have a lower optimal temperature for depuration than those grown at warmer temperatures (Presnell and others 1969; Neilson and others 1978). Loosanoff (1958) studied effects of water temperature on pumping rate of Gulf oysters (*Crassostrea virginica*) through their gills using kymograph and reported that only insignificant quantities of water were pumped by the oysters at temperatures below 3°C. The pumping appeared to increase rapidly from 8 °C to 16 °C with no further increase observed when temperatures were increased from 16 to 28 °C. Souness and Fleet (1979) evaluated the effects of water temperature on depuration of Sydney rock oyster (*Crassostrea commercialis*) using a dye uptake procedure and reported a water temperature of 25°C was optimal for pumping by oysters. The pumping rates reduced when water temperatures were decreased to 15°C or lower, or increased to 30°C or higher. Table 1.4 lists the optimal temperatures for reducing various organisms in the hard clam, eastern oyster, soft clam, and the Sydney rock oyster by depuration.

1.7.2 Salinity

The salinity of the seawater used for depuration of molluscan shellfish is of critical importance. The effect of salinity fluctuation by tidal cycle on oyster pumping has

Table 1.4 Optimal temperatures for reducing various types of microorganisms in shellfish by depuration (Data adapted from Roderick and Schneider 1994)

	Optimal Temperature (°C) for Depuration
Hard Clam	
Fecal coliform	22-25
E. coli	20
V. parahaemolyticus	15
V. cholerae	22-24
V. vulnificus	22-24
Polio type 1	20
Polio type 3	18-20
Coxsackie B4	20
Eastern oyster	
Fecal coliform	24-29
Total coliform	24-29
V. cholerae	22-24
V. vulnificus	22-24
Polio type 1	12-23
Polio type 3	18-20
Coxsackie B4	20
Hepatitis A	12-23
Sydney Rock Oyster	
Total coliform	18-20
E. coli	13-20
Salmonella spp.	18-22
Soft-shelled Clam	
Total coliform	8-16
E. coli	8-16
Salmonella spp.	13

been reported (Korringa 1952; Roderick and Schneider 1994). The oysters appeared to feed at any time at a high salinity but stop the feeding activity at a low salinity. Therefore, higher salinities seem to enhance the depuration process while lower salinities reduce the efficacy of the process. Since the salinity of water used for depuration may differ from the area that shellfish is harvested, shellfish should be acclimated to their new seawater environment prior to the depuration processing. The period of acclimation varies but should be long enough to allow for adequate pumping activity to restore. It is recommended that the salinity of the depuration water does not differ by 20 % than that of the water where shellfish were harvested (NSSP 1990)

1.7.3 Dissolved Oxygen

Molluscan shellfish require oxygen to maintain normal physiological activities (Roderick and Schneider 1994). The oxygen concentration in water for depuration is limited by the solubility of oxygen in seawater, which usually decreases with the rise of temperature and with an increase in the salinity of the water. It is recommended that the oxygen level should not fall below 2 mg/L in seawater during depuration.

1.7.4 pH of Seawater

Oysters live in brackish water the pH of which is usually above 7.0. The pH range for normal growth was 6.75 to 8.75 for oyster larvae and the rate of growth dropped rapidly at pH levels below 6.75 (Calabrese and Davis 1966). The pH value of water could affect the oyster's pumping activity. It has been reported that a low pH value, which might occur naturally by an influx of acid swamp water or by industrial pollution, could

reduce the oyster's pumping rate. Loosanoff and Tommers (1947) found that oyster pumped normally at pH of 7.75, but the rate of pumping decreased when pH dropped to 6.5 and further decreased to 10 % of its normal pumping rate when pH decreased to 4.14.

1.7.5 Turbidity and total Suspended solids

Mollusan shellfish are filter feeders and most of the suspended solids in the seawater can be filtered from the seawater. Loosanoff and Tommers (1948) studied effects of silt and other turbidity–creating substances on the rate of pumping and found that low degrees of suspended solids (0.1 g/L) in seawater could reduce oyster's pumping rate to 40 % of the normal value. The pumping rate of the Gulf oysters (*Gryphaea virginica*) was further reduced to 4% of the normal quantity if the suspended solids increased to 3-4 g/L in seawater.

Through the filter-feeding activity, oysters expel large quantities of the suspended materials named pseudofeces, which are commonly referred as "bio-deposits" and are a reservoir of bacteria that can recontaminate the shellfish during deputation process (Roderick and Schneider 1994). These deposits constitute a potential bacterial and toxic hazard. If the water flow is stopped during the depuration, the levels of dissolved oxygen in the water can drop quickly and hydrogen sulfide created through decomposition of these deposits could kill the shellfish in the depuration tank. In addition, excessive turbidity will reduce the penetrating power of the ultraviolet light used to disinfect the seawater. Therefore, a filter device should be used to remove the bio-deposits in the seawater in a re-circulating depuration system.

1.8 Improving efficacy of depuration

To increase the efficacy in reducing bacterial contamination in oysters, depuration in conjunction with chlorine, ultraviolet light, ozone or iodophors were studied (Fleet 1978). However, none of them could effectively eliminate *V. parahaemolyticus* from shellfish. Croci and others (2002) studied depuration of blue mussels experimentally contaminated with *Escherichia coli*, *Vibrio cholerae* and *V. parahaemolyticus* in ozonated water and reported a substantially smaller reduction in the numbers of *V. cholerae* and *V. parahaemolyticus* in the mussels (approximately 1 log) than of *E. coli* (approximately 3 log) after 44 h of process. Ren and Su (2006) examined the effects of electrolyzed oxidizing (EO) water depuration on reducing *V. parahaemolyticus* and *V. vulnificus* in laboratory-contaminated oysters and found that both species could only be reduced by approximately 1.0-log unit after 8 h at room temperature.

It is now well known that depuration at ambient temperature would not be an option for effective reduction of *V. parahaemolyticus* in oysters. However, decreasing water temperature during the process may turn the process into an effective means for decontaminating *V. parahaemolyticus* in oysters. Studies of occurrence of *V. parahaemolyticus* in the marine environments have demonstrated that densities of *V. parahaemolyticus* in seawater were positively correlated with water temperatures (Kaneko and Colwell 1973; DePaola and others 1990; Duan and Su 2005). Schwarz (2000) reported that a rapid chilling of shellstock oysters to 1.6°C in an ice waterbath resulted in an average of 97.8% reduction of *V. vulnificus* in the oysters. These reports indicate that low temperature appears to be a key factor affecting densities of *V parahaemolyticus* in the marine environments and in oysters.

This study was conducted to investigate the impacts of low-temperature on increasing efficacy of depuration for reducing *V. parahaemolyticus* and *V. vulnificus* contamination in raw oysters.

1.9 Electrolyzed oxidizing (EO) water

Electrolyzed oxidizing (EO) water is produced by the electrolysis of the dilute (ca.0.1%) sodium chloride (NaCl) solution utilizing a commercially available electrolysis apparatus. The electrolysis can be conducted with a low level of 10-20V of DC using either a single-cell chamber or the two-cell chamber separated by a diaphragm (Venczel and others 1997). Using the two-cell chamber, the generation of EO water involves reaction in a cell containing inert positively charged and negatively charged electrodes, respectively, separated by a membrane, and through which a very dilute salt water solution passes (Kim and others 2000b). By subjecting the electrodes to direct current voltage, two types of water possessing different characteristics are generated (Figure 1.1). An electrolyzed basic aqueous solution (pH 11.4 and oxidation-reduction potential [ORP] -795mV) is produced from the cathode side and has a reducing potential that leads to a reduction of free radicals in biological systems. Strongly acidic electrolyzed water (AcEW) containing hypochlorous acid (HOCl) and dissolved chlorine gas is produced in the anode compartment (Nakagawara and others 1998; Suzuki and others 2002).

The AcEW normally has a pH of 2.7 or lower with an oxidation-reduction potential (ORP) of greater than 1,100 mV and a free-chlorine concentration of 10 to 80 ppm (Shimizu and Hurusawa 1992). The concentration of HOCl produced in the AcEW is dependent on the concentration of NaCl used and can be adjusted by changing the

amperage and voltage used for the electrolysis (Len and others 2000; Hsu 2003). A major advantage of using EO water for inactivation of bacteria is that it is produced using pure water and NaCl and can be generated on-site without handling any hazardous chemicals (White 1999). Therefore, it has less adverse impact on the environment and is safer for workers.

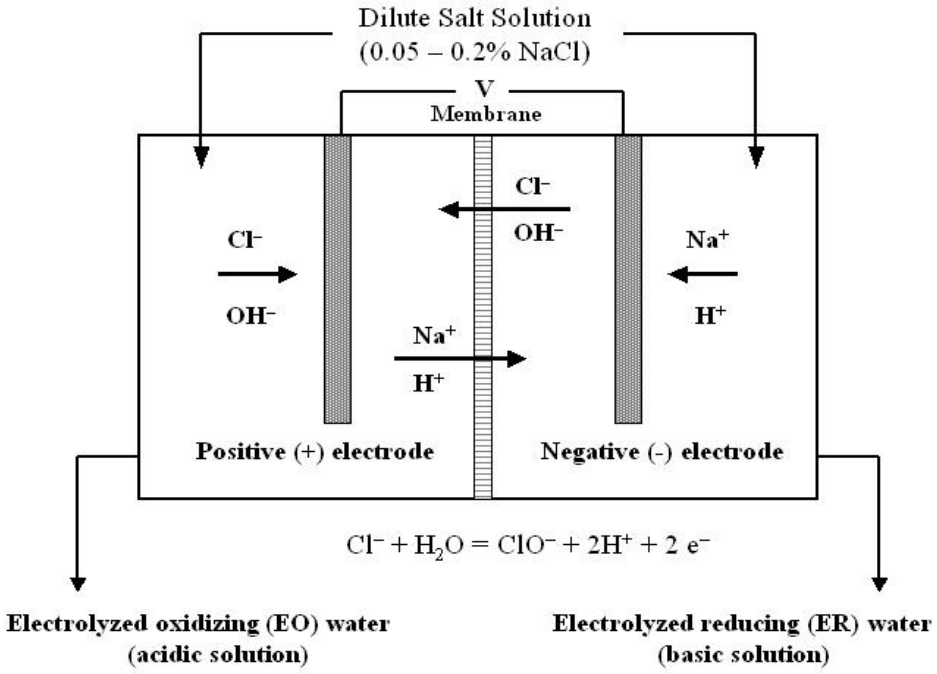

Figure 1.1 Schematic of EO water generator and produced compounds

1.9.1 Antibacterial properties of EO water

EO water has been reported to exhibit strong antibacterial activities against many foodborne pathogens, including *E. coli* O157:H7, *Salmonella* Enteritidis, *Listeria monocytogenes, Bacillus cereus, Campylobacter jejuni, Vibrio parahaemolyticus* and

Vibrio vulnificus (Table 1.5) (Kim and others 2000a; Kim 2001; Park and others 2001, 2002; Ren and Su 2006). Application of EO water as disinfectant for reducing microbial contaminations has been reported for fresh fruits and vegetable (Izumi 1999; Koseki and Itoh 2001; Park and others 2001; Koseki and others 2002; Deza and Garrido 2003), poultry carcasses (Fabrizio and others 2002; Park and others 2002; Kim and others 2005), eggshell (Park and others 2005), and seafood (Mahmound and others 2004;Loi-Braden and others 2005; Huang and others 2006; Ren and Su 2006).

The active bactericidal agents of EO water have been reported to be related to its contents of hypochlorous acid (HOCl), Oxidation-Reduction Potential (ORP), and low pH (Kim and others 2000a; Len and others 2000). Marriott (1985) reported that HOCl existing in chlorinated water is responsible for the lethal reactions associated with the bacterial cell membrane, DNA denaturation, or disruption of protein synthesis. Albrich and Hust (1982) noted that the microbial action of HOCl arises from interruption of energy-linked cellular respiration due to bacterial cells exposed to HOCl underwent irreversible oxidation of cytochrome b, carotene, and adenine nucleotides. Other researchers also reported that HOCl could cause profound alteration of microbial cell membrane metabolite transport capabilities and denaturation of enzymes on cell surfaces and lowering cytoplasmic pH values due to proton permeation through membrane in response to the increased pH gradient (Venkobachar and others 1977; Camper and Mcfeters 1979).

The high ORP value is an important factor contributing to the antibacterial activity of EO water. Most aerobic bacteria require a positive ORP (+200 to +800 mV) while anaerobic bacteria need a negative ORP (-40 and -400 mV) to grow.

Table 1.5 Inactivation of *E. coli* O157:H7, *Listeria monocytogenes*, *Bacillus cereus* and *Campylobacter jejuni* by EO water (Data adapted from Kim and others 2000a and Park and others 2002)

Bacterial Species	Time(s)	Surviving Population (log_{10}CFU/ml)			EO water property		
		Initial Population	Control[a]	EO water	pH	ORP	Free Chlorine (ppm)
E. coli O157:H7	10s	9.91	9.62	NE[d]	2.6	1,160	56
	30s	10.25	9.71	NE			
L. monocytogenes	10s	10.21	9.98	NE			
	30s	10.11	10.09	NE			
B. cereus[b]	10s	7.75	7.62	4.42			
	30s	8.47	8.11	NE			
B. cereus[c]	30s	8.03	7.95	6.45			
	120s	6.93	6.96	3.44			
C. jejuni	10s	7.47	7.48	NE	2.57	1,082	51.6
	30s	7.47	7.33	NE			

[a] Deionized water
[b] *B. cereus* vegetative cells
[c] *B. cereus* spores
[d] Negative in enrichment

Hence, the high ORP (ca. 1,150 mV) of EO water creates an environment that is not suitable for growth of bacteria. Early in 1968, a German Federal Health Office Laboratory first proved that the killing rate of *E. coli* was dependent on ORPs and not on residual chlorine (McPherson 1993). Since then, similar findings have been observed killing of bacteria was not totally based on a defined chlorine reaction and that higher ORPs were required for complete killing of *E. coli* (Carlson 1991; Robbs and others 1995). Kim and others (2000b) compared the pH, ORP, and residual chlorine of EO water for inactivating *E. coli* O157:H7 and found that both ORP and residual chlorine contributed to bacterial inactivation. The results suggested that the high ORP plays an important role in EO water's antibacterial properties.

The low pH of EO water has been reported to be a minor factor contributing to EO water's antimicrobial property. The pH of EO water is usually around 2.5 and, therefore, has inhibitory effect on growth of many bacteria due a low pH that is below the normal growth range of bacteria. Although studies have indicated that both chlorine and high ORP value are more important than low pH in contributing to EO water's antibacterial activity, it is very clear that EO water would not exhibit the strongest bactericidal effects without available chlorine, high ORP, and low pH value.

1.9.2 Application of EO water

EO water was first developed in Japan and in by medical and dental professionals for treating wounds or disinfecting medical equipments (Shimizu and Hurusawa 1992). Today, the technology has been utilized in various fields in agriculture, livestock management, and areas that rely on bacterial disinfection

(Wullaert 1997). Recently, EO water has received a great attention in the food industries as an alternative to chlorine for reducing microbial contamination.

1.9.2.1 Vegetable and fruits

Fruits and vegetables can become contaminated with pathogenic micro-organisms while growing in fields, during harvesting and postharvest handling, processing and distribution (Beuchat 1995). Washing fresh produce with running tap water may remove soil and other debris, but has a limited effect on removing surface microorganisms (Koseki and Itoh 2001).

Koseki and Itoh (2001) reported that washing fresh-cut lettuce and cabbage in EO water not only resulted in a decrease of initial microbial populations but also retarded growth of the bacteria in the products when stored at refrigeration temperatures. It took about 3 days to allow the bacteria to grow to the initial population when the products were stored at 10°C. Park and others (2001) also reported that washing lettuce leaf with EO water for 3 min significantly ($p<0.05$) reduced population of inoculated *E. coli* O517:H7 and *L. monocytogenes* by 2.41 and 2.65 log CFU/g, respectively, without creating significant changes in quality of washed lettuce during 2 weeks of storage.

In addition to acidic EO water, neutral electrolyzed water (NEW) with a near neutral pH was recently developed by redirecting the electrolyzed water formed at the anode chamber to the cathode chamber during the water electrolysis. Izumi (1999) found that NEW (pH 6.8, chlorine content: 20 ppm) was effective in reducing bacterial contamination (0.6 to 2.6 logs CFU/g reductions) on fresh-cut vegetables without

causing discoloration. Washing tomato with NEW was capable of reducing inoculated bacteria (*E. coli* O157:H7, *Salmonella* enteritidis, *L. monocytogenes*, and non-pathogenic *E. coli*) on the surfaces from about 5 log CFU/cm^2 to <1 CFU/cm^2 (Deza and Garrido 2003).

In addition to its liquid form, the bactericidal effect of EO water was also observed in ice form. Populations of aerobic bacteria on lettuce was reduced by 1.5 log CFU/g when lettuce was stored in ice made of EO water and kept at 2-3°C for 24 h (Koseki 2002). The use of ice made of EO water offers an added benefit by providing a low temperature storage condition and inactivation of bacteria at same time.

1.9.2.2 Eggshell

It is estimated that 7.8% of eggs were contaminated with *Salmonella* on the shell before washing and approximately 1.1% of washed egg remained contaminated with the bacterium (Humphrey 1994). Park and others (2005) studied the effect of washing shell eggs with EO water and reported that washing the eggs with alkaline EO water for 1 min followed by another wash with acidic EO water (41 ppm chlorine) for 1 min was capable of reducing *Listeria* and *Salmonella* by 4.39 and 3.66 log CFU/ Shell egg, respectively. The reductions were similar to those (4.01-log reduction for *Listeria* and 3.81-log reduction for *Salmonella*) obtained by washing the eggs with chlorinated water with a much higher chlorine concentration of 200 ppm for 1 min.

1.9.2.3 Poultry

Raw poultry products have been perceived to be responsible for a large number of human illnesses because of high frequencies of contamination with pathogen such as *Campylobacter jejuni* in poultry ((Humphrey and others 1993). Recent study has shown that washing chicken wings inoculated with *C. jejuni* (5.05 log CFU/g) with EO water for 10 min at 23°C resulted in a reduction of the bacterium by 3 log CFU/g, whereas washing the wings with deionized water (control) only resulted in 1-log CFU/g of reduction (Park and others 2002). Fabrizio and others (2002) investigated effects of EO water on reducing *Salmonella* Typhimurium on poultry and found that a treatment of EO water (pH 2.6, chlorine: 20-50 ppm, ORP: 1,150 mV) at 4°C for 45 min following refrigerated storage could reduce *S.* Typhimurium on poultry surfaces. Populations of *S.* Typhimurium on broiler carcasses (2.71 log CFU/ml of rinsate) were reduced by 0.83 log CFU/ml after the EO water treatment and to nearly undetectable levels after 7 d of storage at 4°C. Kim and others (2005) evaluated the efficacy of alkaline electrolyzed reducing water (ER water) and EO water in preventing and removing fecal contaminants and killing *C. jejuni* on poultry carcasses under simulated industrial processing conditions. They found that ER water could be used to replace trisodium phosphates (TSP) in preventing attachment and removal of feces on surfaces of chicken carcasses. Immersion chicken carcasses in EO water significantly reduced the population of *C. jejuni* by 2.33 log CFU/g.

1.9.2.4 Seafood

Fresh seafood normally has a short shell life because of spoilage caused by growth of bacteria commonly harbored on skin and gill. It is fairly common that the

seafood industry use chlorinated water to rinse seafood during processing in order to reduce microbiological contamination and increase product shelf life (Huss 1994). EO water has been reported to be capable of reducing bacterial contamination on fish. Washing shrimp with EO water (40 ppm available chlorine) effectively removed *E. coli* O157:H7 and *Salmonella* spp. from the shell surface (Loi-Braden and others 2005). Shrimp treated by the EO water had significantly lower bacterial loads than untreated controls and showed no difference in sensory attributes when compared with those treated with tap water or aqueous chlorine

Mahmoud and others (2004) studied the effects of EO water on reducing total bacteria count on fresh whole and skinless filleted carp and found that dipping whole carp in deionized water at 25°C for 15 min could only reduce total microbial count on skin by 0.6 log CFU/cm^2. However, the same treatment with EO water (pH 2.22, chlorine: 40 ppm, ORP: 1,137 mV) resulted in 2.6 log CFU/cm^2 reduction of total microbial count. Huang and others (2006) studied EO water for reducing microbial population on tilapia and platform of fish retailer. They reported that EO water achieved additional 0.7 log CFU/cm^2 reduction of *E. coli* than tap water after 1 min of immersion treatment. The EO water treatments also reduced *V. parahaemolyticus* on tilapia by 1.5 and 2.6 log CFU/cm^2 after 5 and 10 min, respectively. In addition, treatments of EO water also effectively disinfected the platform of fish retailer in traditional and fish markets.

Recently, Ren and Su (2006) reported that EO water exhibited strong bactericidal effects against *V. parahaemolyticus* and *V. vulnificus*. Holding Pacific Oysters inoculated with *V. parahaemolyticus* or *V. vulnificus* in the EO water

containing 1% NaCl for 4 to 6 h at room temperature was capable of reducing *V. parahaemolyticus* and *V. vulnificus* in the oyster by greater than 1.0 log MPN/g.

In summary, EO water has been demonstrated to be an effective disinfecting agent and can be used as a chlorine alternative for reducing microbial contamination on raw materials and processing surfaces. The main goal of this study was to determine the temperature effects on artificial seawater and EO water depuration for reducing *V. parahaemolyticus* and *V. vulnificus* contamination in raw oysters.

Chapter II

Impact of Low-temperature Depuration on *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Gulf Oyster (*Crassostrea virginica*)

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2.1 ABSTRACT

This study investigated effects of low-temperature depuration on reducing *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the Gulf oyster. Raw Gulf oysters were inoculated with a five-strain cocktail of *V. parahaemolyticus* or *V. vulnificus* at levels of 10^{4-5} MPN (most probable number)/g and depurated with artificial seawater (ASW) or electrolyzed oxidizing (EO) water (chlorine, 30 ppm; pH 2.82; oxidation-reduction potential, 1,131mV) containing 3% NaCl at 22, 15, 10 and 5°C. Reduction of *V. parahaemolyticus* and *V. vulnificus* in oyster by EO water depuration at 22°C for 48 h was limited to 0.7 and 1.4 log MPN/g, respectively. Depuration with EO water at lower temperature had little impact on enhancing the reduction of *Vibrio* in the oyster. Greater reductions of *V. parahaemolyticus* (1.2 log MPN/g) and *V. vulnificus* (2.0 log MPN/g) in the Gulf oyster were achieved after 48 h of ASW depuration at 22°C. Decreasing ASW depuration temperature to 15°C increased the reductions of *V. parahaemolyticus* and *V. vulnificus* to 2.1 and 2.9 log MPN/g after 48 h of treatments, respectively. However, depuration of oyster in ASW at 10 and 5°C were found less effective in reducing the *Vibrio* spp. in the Gulf oyster. An extended depuration with ASW at 15°C for 96 h was capable of achieving greater than 2.5 log MPN/g of reductions of both *V. parahaemolyticus* and *V. vulnificus* in the Gulf oyster.

.2.2 INTRODUCTION

Vibrio parahaemolyticus and *Vibrio vulnificus* are human pathogens that occur naturally in the marine environment and are frequently isolated from raw seafoods, particularly oyster. These organisms are the major causes of seafood-borne infections in the United States with *V. parahaemolyticus* being the leading cause of acute gastroenteritis associated with seafood consumption (Levine and Griffin 1993; Rippey 1994; Mead and others 1999). In addition to its capability of causing gastroenteritis, *V. vulnificus* can also cause wound infection and infect the bloodstream, which may lead to development of a severe and life-threatening illness (septicemia) in immunocompromised persons, particularly those with chronic liver disease, with a high fatal rate of 50% (CDC 2005b).

It is estimated that 20 million Americans eat raw shellfish and one in 2,000 meals of raw molluscan shellfish serves as the vehicle for *Vibrio* infection, making shellfish one of the most hazardous seafoods (Ahmed 1991; Rippey 1994). Consumption of raw and undercooked shellfish has been reported to account for more than 90% of 113,000 seafood-poisoning cases each year in the U.S. (Mead and others 1999). Numerous outbreaks of *Vibrio* infections linked to raw oyster consumption have been reported in the U.S. over the past ten years (CDC 1998, 1999, 2006b; DePaola and others 2000; McLaughlin and others 2005). The U.S. Centers for Disease Control and Prevention recently reported a 78% increase in incidence of *Vibrio* infections in 2006 from the 1996-1998 baseline, indicating further measures are needed to prevent *Vibrio* infections (CDC 2007).

The density of *V. parahaemolyticus* and *V. vulnificus* in oyster increases with water temperature reaching typically the highest value in the summer months (DePaola and others 1990; Motes and DePaola 1996). Although rapid cooling to refrigeration temperature can effectively prevent growth of *V. parahaemolyticus* and *V. vulnificus* in oyster post harvest, exposure of oyster to elevated temperatures during transportation and retail storage cannot be totally avoided. A survey of 370 lots of oysters sold at restaurants, oyster bars, retail and wholesale seafood markets throughout the U.S. found that densities of *V. parahaemolyticus* and *V. vulnificus* in Gulf oysters were generally 10- to 100-fold greater at retail than those observed at harvest (Cook and others 2002). A post-harvest process that can effectively reduce *Vibrio* contamination in raw oyster could enhance the safety of raw oyster consumption.

Depuration is the process of holding filter-feeding shellfish in clean seawater to allow them to purge sand and bacteria (Blogoslawski and Stewart 1983). While the process has a long history as a post-harvest treatment to reduce microbial contaminants in shellfish, it is ineffective in reducing *Vibrio* contamination when conducted at room temperature (Colwell and Liston 1960; Vasconcelos and Lee 1972). Kelly and Dinuzzo (1985) showed that laboratory-contaminated oysters required 16 days to depurate *V. vulnificus* to non-detectable level. The retention and colonization of *Vibrio* spp. within shellfish limits the use of conventional depuration as a means for eliminating the contaminant. However, replacing clean seawater with a solution exhibiting bactericidal activity or decreasing water temperature for the depuration process may increase the efficacy of the process for decontaminating *Vibrio* spp. in shellfish.

Electrolyzed oxidizing (EO) water generated through electrolysis of a dilute salt solution has been reported to exhibit strong antibacterial activities against many foodborne pathogens, including *Escherichia coli* O157:H7, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Bacillus cereus*, *Campylobacter jejuni*, *V. parahaemolyticus*, and *V. vulnificus* (Kim and others 2000; Kim and others 2001; Park and others 2001; Park and others 2002; Ren and Su 2006). Application of EO water as disinfectant for reducing microbial contaminations has been reported for fresh fruits and vegetable (Izumi 1999; Koseki and Itoh 2001; Park and others 2001; Koseki and others 2002; Deza and Garrido 2003), poultry carcasses (Fabrizio and others 2002; Park and others 2002; Kim and others 2005), eggshell (Park and others 2005), and seafood (Mahmound and others 2004; Loi-Braden and others 2005; Huang and others 2006; Ren and Su 2006). This study was conducted to determine impact of low-temperature EO water or ASW depuration on *V. parahaemolyticus* and *V. vulnificus* in Gulf oyster.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial cultures preparation. *V. parahaemolyticus* (10290, 10292, 10293, BE 98-2029, and 027-1c1) and *V. vulnificus* (93A3097, 93A4153, 96A6135, ATCC27562, and DI27-3C) were used in this study. All strains, except *V. vulnificus* ATCC27562 and *V. vulnificus* DI27-3C (isolated from oyster), were clinical isolates obtained from the collection of the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, Wash.). Each culture was individually grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Spark, Md.) supplemented with 1.5% NaCl (TSB-Salt) at 37°C for 18 to 24 h. The cultures were streaked to individual tryptic soy agar (TSA; Difco,

Becton Dickinson) supplemented with 1.5% NaCl (TSA-Salt) and incubated at 37°C for 18 to 24 h. A single colony was then selected from the TSA-salt plate and enriched in TSB-Salt at 37°C for 4 h. The enriched cultures of *V. parahaemolyticus* or *V. vulnificus* were pooled into a sterile centrifuge tube and centrifuges at 3,000×g (Sorvall RC-5B, Kendro Laboratory Products, Newtown, Conn.) at 5°C for 15 min. Pelleted cells were resuspended in 50 ml of sterile salt solution (1%) to produce a culture cocktail of approximately 4.1×10^{8} CFU/ml.

2.3.2 EO water production. EO water (chlorine: 30 ppm, pH 2.82, oxidation-reduction potential [ORP]: 1,131 mV) was produced with an electrolyzed water generator (model V-500, Electric Aquagenics Unlimited, Inc., Lindon, Utah) according to manufacturer's instruction. EO water was produced on the day of experiments and used within 10 min after production.

2.3.3 Oyster preparation. Raw Gulf oysters were obtained from a shellfish farm in Louisiana and shipped to the laboratory overnight in a cooler with ice gels. The oysters were washed briefly with tap water to remove mud on the shell and placed in two rectangular high-density polyethlylene (HDPE) tanks (18 by 12 by 12 in,; Nalge, Rochester, N.Y.) containing artificial seawater ([ASW] salinity: 30 ppt) at room temperature for 3 to 4 h before being inoculated with *Vibrio* spp. The ASW was prepared by dissolving Instant Ocean Salt (Aquatic Eco-System, Inc., Apopka, Fla.) in deionized water according to manufacturer's instruction.

2.3.4 Inoculation of oysters with *Vibrio* spp. For each experiment, 80 oysters were transferred from the ASW to two similar HDPE (forty each) of fresh ASW containing *V. parahaemolyticus* (or *V. vulnificus*) culture cocktail at a level of approximately 10^{4-5}

CFU/ml. The inoculation was conducted at room temperature overnight (12 to 14 h), with water being circulated at a flow rate of 11 liters/h. Air was pumped into the solution to facilitate colonization of *Vibrio* in oysters. Oysters were analyzed for *V. parahaemolyticus* or *V. vulnificus* contamination with a three-tube most-probable-number (MPN) method before the inoculation.

2.3.5 Oyster depuration. Inoculated oysters were divided into two group (40 oysters each) and depurated with 40 liters of ASW or EO water (30 ppm chlorine) containing 3% NaCl in two identical laboratory-scale re-circulating (25L/min) systems (Figure 2.1) each equipped with a 15 W Gamma UV sterilizer (Current-USA Inc., Vista, Calif.), and a water chiller (Delta Star, Aqua Logic, Inc., San Diego, Calif.) at four temperatures (22, 15, 10 and 5°C). Populations of *V. parahaemolyticus* or *V. vulnificus* in oysters were analyzed at 0, 6, 12, 24, 36, and 48 h during the processes. Both ASW and EO water were replaced with freshly prepared solutions at each sampling time to remove suspended solids in the water. Total chlorine in EO water was determined immediately after the water was generated with a commercial chlorine detection kit (HACH Company, Loveland, Colo.). The pH and ORP of EO water were measured with a pH meter (model 420A, Orion Research, Inc., Boston, Mass.) and an ORP meter (CheckmateII Systems with Redox Sensor, Corning, Inc., Corning, N.Y.), respectively. The most effective treatment for reducing *V. parahaemolyticus* and *V. vulnificus* in oysters was chosen to determine if a longer treatment time (96 h) with sampling every 12 h would result in additional reduction of both *V. parahaemolyticus* and *V. vulnificus* in oysters.

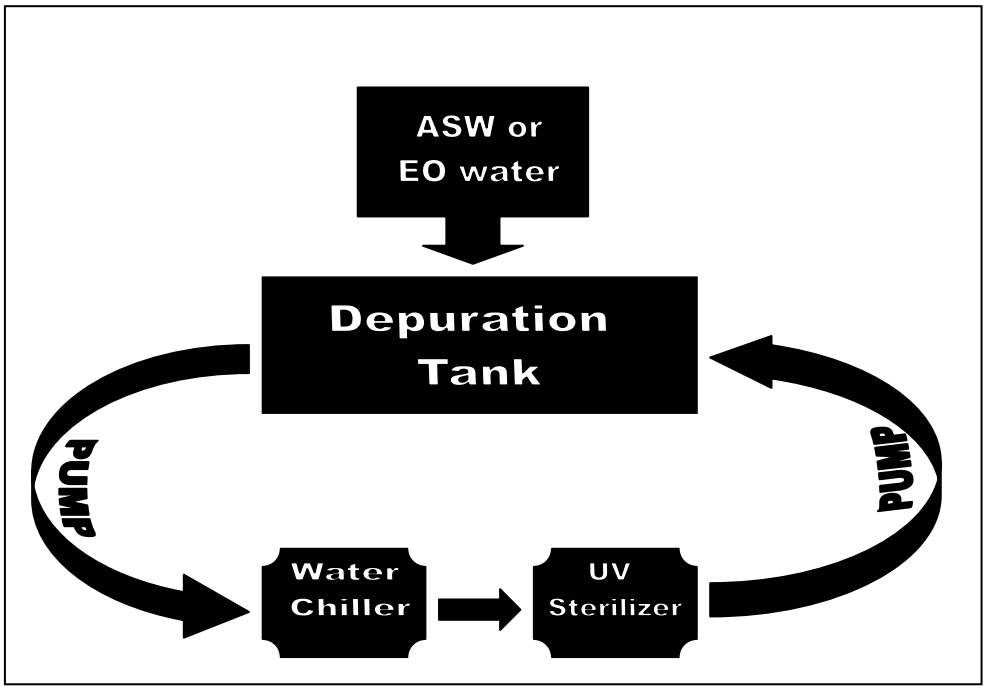

Figure 2.1 Schematic of Oyster Depuration System

2.3.6 Microbiological tests. Populations of *V. parahaemolyticus* or *V. vulnificus* in inoculated oysters during each depuration study were analyzed with a three tube MPN method described in the U.S. Food and Drug Administration's Bacteriological Analytical Manual (USFDA 1998), using thiosulfate-citrate-bile salts-sucrose agar (TCBS) for *V. parahaemolyticus* or modified cellobiose polymyxin colistin agar (mCPC) for *V. vulnificus* determination. At each testing time, four oysters were randomly picked from each treatment and shucked with a sterile shucking knife in a sterile stainless steel tray. Each shucked oyster meat was placed in a sterile blender jar and blended with 9 volume of sterile alkaline peptone water (APW) at high speed for 1 min using a two-speed laboratory blender (Waring Laboratory, Torrington, Conn.) to prepare a 1:10 dilution sample suspension. Two additional 10-fold dilutions for each oyster sample were prepared with sterile APW. All sample dilutions were individually inoculated into three tubes of alkaline peptone salt broth (APS). Inoculated APS tubes were incubated at 35 to 37°C for 16 to 18 h and one loopful (3mm) of enriched APS from a turbid tube was streaked onto individual TCBS for *V. parahaemolyticus* detection, or onto mCPC for *V. vulnificus* detection. All plates were incubated at 35 to 37°C for 18 to 24 h. Formation of colonies that are round (2- to 3-mm diameter) and green or bluish on TCBS or colonies that are round (1- to 2-mm diameter), flat, and yellow on mCPC was considered positive for *V. parahaemolyticus* or *V. vulnificus*. Total populations of *V. parahaemolyticus* or *V. vulnificus* in oysters were determined by converting numbers of APS tubes that were positive for *V. parahaemolyticus* or *V. vulnificus* to MPN/g using an MPN table. Results were reported as means of four determinations.

2.3.7 Statistical analysis. Results of microbiological tests were transformed into log values for statistical analyses. Bacterial populations in oysters at different treatment times were analyzed with two-sample t test (S-plus, Insightful Corp., Seattle, Wash.). Significant differences between means of treatments were established at a level of $P = 0.05$.

2.4 RESULTS AND DISCUSSION

2.4.1 Effects of temperatures on EO water depuration for reducing *Vibrio* contamination in oysters. Changes of *V. parahaemolyticus* in oyster depurated with EO water at various temperatures are shown in Table 2.1. Previous study has reported that EO water depuration at room temperature for 4-8 h could reduce *V. parahaemolyticus* in the Pacific oyster (*Crassostrea gigas*) by approximately 1.0 log MPN/g (Ren and Su 2006). This study found that the population of *V. parahaemolyticus* in the Gulf oyster could only be reduced by about 0.8 log MPN/g after 12 h of EO water depuration at 22°C. Reducing water temperature to 15°C for depuration did not enhance the reduction of *V. parahaemolyticus* in the oyster. Depuration in EO water at 15°C for 48 h only resulted in 0.8 log MPN/g of reduction of the organism in oyster. No significant reductions of *V. parahaemolyticus* in oyster were observed with EO water depuration at 10 or 5°C.

Similar results were obtained for *V. vulnificus* in oysters depurated in EO water at various temperatures (Table 2.2). Depuration with EO water at 22°C for 48 h resulted in 1.4 log MPN/g of reduction of *V. vulnificus* in the Gulf oyster. The reduction of *V.*

Table 2.1 Changes of *Vibrio parahaemolyticus* (Log MPN/g) in laboratory-inoculated Gulf oysters depurated with salt-containing electrolyzed oxidizing water at various temperatures

Processing time (h)	Temperature (°C)			
	22	15	10	5
0	5.40 ± 0.43[a] A	5.93 ± 0.34 A	4.39 ± 0.70 A	5.16 ± 0.21 A
6	4.74 ± 0.60 (0.66)[b] AB	5.62 ± 0.87 (0.31) AB	4.01 ± 0.25 (0.38) A	5.14 ± 0.86 (0.02) A
12	4.61 ± 0.32 (0.79) B	5.77 ± 0.43 (0.16) AB	4.23 ± 0.47 (0.16) A	4.86 ± 0.55 (0.30) A
24	4.54 ± 0.24 (0.86) B	6.12 ± 0.34 (0.00) AB	4.02 ± 0.74 (0.37) A	4.76 ± 0.48 (0.40) A
36	4.72 ± 0.49 (0.68) AB	5.30 ± 0.68 (0.63) AB	4.12 ± 0.34 (0.27) A	4.87 ± 0.43 (0.29) A
48	4.71 ± 0.77 (0.69) AB	5.09 ± 0.51 (0.84) B	3.95 ± 0.19 (0.44) A	4.70 ± 0.47 (0.46) A

[a] Mean of four determinations ± standard deviation. Means with the same letter in the same column are not significantly different (P>0.05).
[b] Reduction (Log CFU/g) of *V. parahaemolyticus* after treatment.

Table 2.2 Changes of *Vibrio vulnificus* (Log MPN/g) in laboratory-inoculated Gulf oysters depurated with salt-containing electrolyzed oxidizing water at various temperatures

Processing time (h)	Temperature (°C)			
	22	15	10	5
0	5.27 ± 0.43[a] A	5.02 ± 0.24 A	4.77 ± 0.61 A	4.02 ± 0.42 A
6	4.82 ± 0.52 (0.45)[b] AB	4.96 ± 0.42 (0.06) A	4.44 ± 0.87 (0.33) AB	3.94 ± 0.55 (0.08) AB
12	4.44 ± 0.30 (0.83) BC	4.63 ± 0.68 (0.39) A	4.32 ± 1.07 (0.45) ABC	3.82 ± 0.67 (0.20) ABC
24	4.44 ± 0.77 (0.83) ABC	4.02 ± 0.80 (1.00) A	3.96 ± 0.81 (0.81) ABC	3.28 ± 0.12 (0.74) BC
36	4.07 ± 0.30 (1.20) C	3.21 ± 0.20 (1.81) B	3.55 ± 0.52 (1.22) BC	3.06 ± 0.42 (0.96) C
48	3.90 ± 0.63 (1.37) BC	3.20 ± 0.36 (1.82) B	3.17 ± 0.16 (1.60) C	3.17 ± 0.16 (0.85) C

[a] Mean of four determinations ± standard deviation. Means with the same letter in the same column are not significantly different (P>0.05).
[b] Reduction (Log CFU/g) of *V. vulnificus* after treatment.

vulnificus in the oyster increased slightly to 1.8 log MPN/g when oyster was depurated in EO water at 15°C for 48 h. However, further reduction of EO water temperature to 10 or 5°C for depuration resulted in less reductions of *V. vulnificus* in the oyster (1.6 log MPN/g at 10°C and 0.9 log MPN/g at 5°C) after 48 h of process.

Oyster makes rapid biological activity adjustments in response to environment changes such as temperature, pH, and turbidity (Loosanoff 1958; Loosanoff and Tommers 1947, 1948). Loosanoff and Tommers (1947) found that low pH values could reduce the oyster's pumping rate and oyster would reduce the rate to 10% of its normal volume when exposed to an environment of pH 4.1. Ren and Su (2006) reported that oyster could be for forced to stop its water filtering activity upon exposure to EO water because of low pH and chlorine in EO water. Although this study found that depuration of oyster in EO water at 15°C could increase the reduction of the bacterium in oyster, the increase was small (0.4 log MPN/g). Decreasing EO water temperature for depuration did not promote the reduction of *V. parahaemolyticus* or *V. vulnificus* in the Gulf oyster.

2.4.2 Effects of Temperatures on ASW depuration for reducing *Vibrio* contamination in oysters. Changes of *V. parahaemolyticus* in oyster depurated with ASW at various temperatures are reported in Table 2.3. Depuration of contaminated oysters in ASW free of *Vibrio* at 22°C for 48 h resulted in a small reduction (1.2 log MPN/g) of *V. parahaemolyticus* in the oyster with no significant reduction being observed during the first 24 h of process. This result agrees with previous reports that depuration with clean seawater was not effective in reducing *Vibrio* contamination in shellfish (Colwell and Liston 1960; Vasconcelos and Lee 1972; Ren and Su 2006).

However, populations of *V. parahaemolyticus* in oyster were significantly reduced by 1.9 and 2.1 log MPN/g after 24 and 48 h of depuration in ASW at 15°C, respectively. These results seem to indicate that decreasing water temperature for depuration could have a positive impact on reducing *V. parahaemolyticus* in oyster. A further decrease in water temperature to 10°C resulted only in 1.1 and 1.0 log MPN/g of reductions of *V. parahaemolyticus* in oysters after 24 and 48 h, respectively. No apparent reductions of *V. parahaemolyticus* in oysters were observed when the oyster was depurated in ASW at 5°C.

Similar trends in *V. vulnificus* reduction were observed when oysters were depurated with ASW at 5, 10, 15 and 22°C (Table 2.4). However, the reductions of *V. vulnificus* in oyster were all greater than the reductions of *V. parahaemolyticus* observed at each temperature. Depuration of oyster at 22°C for 48 h was capable of achieving 2.0 log MPN/g of reduction of *V. vulnificus* in oyster. The reduction of *V. vulnificus* in oyster increased to 2.9 log MPN/g when oysters were depurated at 15°C for 48 h. However, depurations in ASW at 10 and 5°C were both less effective than at 22°C in reducing *V. vulnificus* in oyster. Reductions of *V. vulnificus* in oyster after 48 h of ASW depuration at 10 and 5°C were limited to 1.7 and 1.2 log MPN/g, respectively. These results indicate that depuration of the Gulf oyster at 15°C for 48 h could increase the reduction of *V. parahaemolyticus* (Figure 2.2) and *V. vulnificus* (Figure 2.3) in oysters by about 1.0 log MPN/g than conventional room temperature depuration. However, reducing water temperature to refrigeration temperature (5-10°C) could not enhance *Vibrio* reduction in contaminated Gulf oyster. Shellfish grown in warm water tend to have an optimal depuration temperature higher than those grown in cold water (Neilson and others 1978).

Table 2.3 Changes of *Vibrio parahaemolyticus* (Log MPN/g) in laboratory-inoculated Gulf oysters depurated with artificial seawater at various temperatures

Processing time (h)	Temperature (°C)			
	22	15	10	5
0	4.83 ± 0.24 [a] A	5.78 ± 0.32 A	4.36 ± 0.47 A	4.72 ± 0.63 A
6	4.65 ± 0.62 AB	5.35 ± 0.62 AB	4.16 ± 0.51 AB	4.26 ± 0.15 A
12	4.20 ± 0.87 ABC	4.80 ± 1.16 ABC	3.82 ± 0.84 AB	4.39 ± 0.32 A
24	4.07 ± 0.50 ABC	3.91 ± 0.84 C	3.26 ± 0.93 AB	4.19 ± 0.43 A
36	3.98 ± 0.22 BC	3.95 ± 1.19 BC	3.39 ± 0.60 B	4.57 ± 0.37 A
48	3.62 ± 0.51 C	3.69 ± 0.27 C	3.31 ± 0.58 B	4.47 ± 0.70 A

[a] Mean of four determinations ± standard deviation. Means with the same letter in the same column are not significantly different (P>0.05).

Table 2.4 Changes of *Vibrio vulnificus* (Log MPN/g) in laboratory-inoculated Gulf oysters depurated with artificial seawater at various temperatures

Processing time (h)	Temperature (°C)			
	22	15	10	5
0	5.42 ± 0.29 [a] A	5.01 ± 0.47 A	4.63 ± 0.50 A	4.35 ± 0.62 A
6	4.28 ± 0.72 B	4.12 ± 0.89 AB	3.87 ± 0.43 AB	3.57 ± 0.37 AB
12	3.38 ± 0.16 BC	3.33 ± 0.10 B	3.19 ± 0.43 BC	3.40 ± 0.20 BC
24	3.53 ± 0.42 BC	3.25 ± 0.42 B	3.32 ± 0.27 BC	3.32 ± 0.26 BC
36	3.12 ± 0.34 C	3.05 ± 0.47 B	2.79 ± 0.85 BC	2.96 ± 0.19 C
48	3.45 ± 0.14 BC	2.13 ± 0.17 C	2.89 ± 0.63 C	3.13 ± 0.17 BC

[a] Mean of four determinations ± standard deviation. Means with the same letter in the same column are not significantly different (P>0.05).

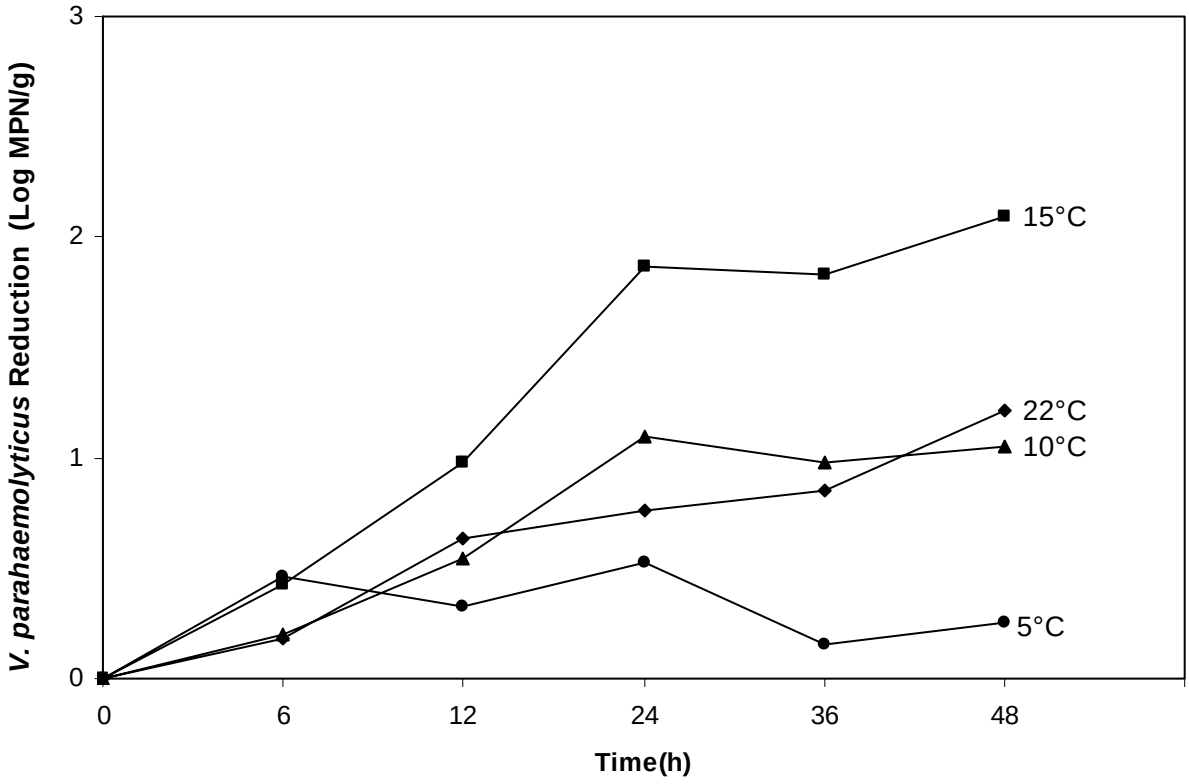

Figure 2.2 Reduction of *Vibrio parahaemolyticus* in laboratory-inoculated oysters depurated in artificial seawater (ASW) at various temperatures

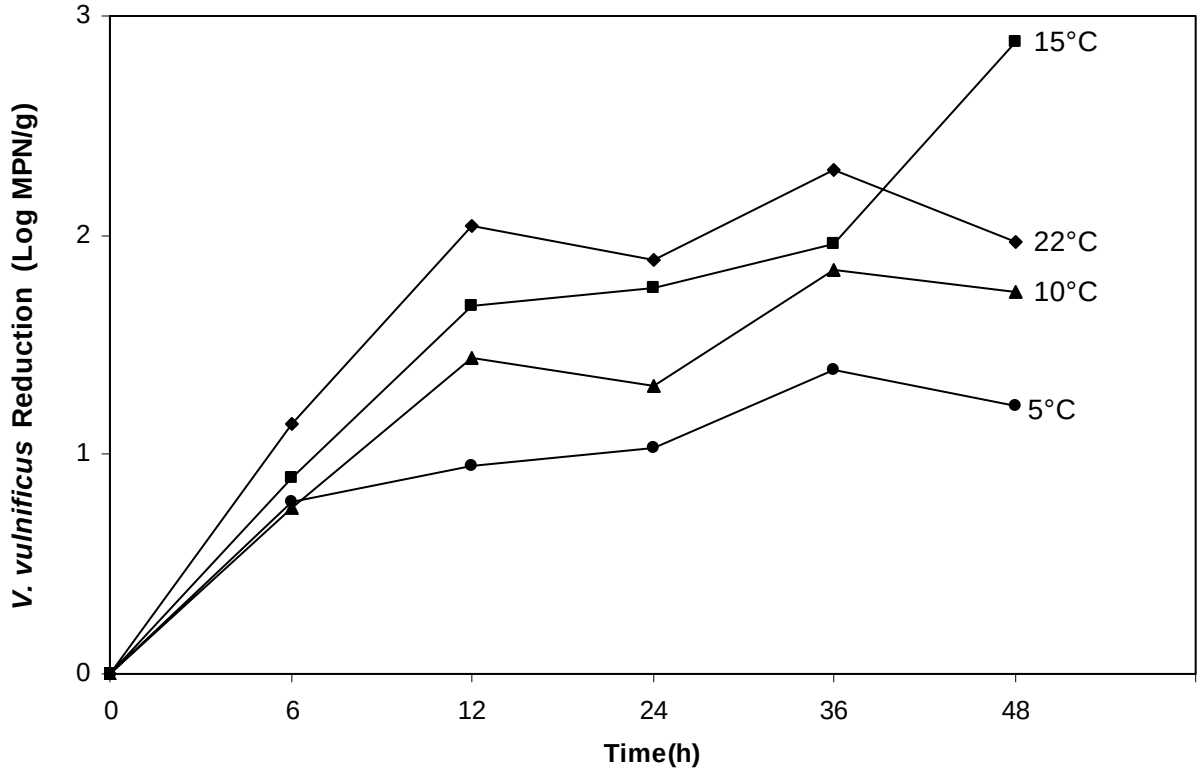

Figure 2.3 Reduction of *Vibrio vulnificus* in laboratory-inoculated oysters depurated in artificial seawater (ASW) at various temperatures

Oyster depuration studies have shown that the water-pumping activity of oyster is affected by water temperature (Souness and Fleet 1979; Rowse and Fleet 1984; Richards 1990; Roderick and Schneider 1994). The Gulf oyster is typically cultivated in the Gulf coast and the Southeast region of the U.S. with water temperature ranging from 50 to 90°F (10-32°C). An early study reported that only insignificant amount of water was pumped by the Gulf oyster when water temperature dropped below 3°C (Loosanoff 1958). However, the pumping activity of the oyster appeared to increase rapidly from 8 to 16°C with no further increase in the activity between 16 and 28°C. These studies suggest that the water-pumping activity of the Gulf oyster would be reduced when the water temperature drops below 10°C and would explain the low depuration levels observed at refrigeration water temperature. It would explain also the increased reductions of *Vibrio* in the Gulf oyster by depuration in ASW at 15°C.

Also important to consider is that low-temperature depuration may limit the growth of *V. parahaemolyticus* and *V. vulnificus*. Both *V. parahaemolyticus* and *V. vulnificus* can multiply rapidly in oysters held at 22°C (Cook and Ruple 1989). Tamplin and Capers (1992) reported that depuration at temperatures higher than 23°C might allow *V. vulnificus* counts to increase in oyster, whereas multiplication of *V. vulnificus* in oyster was limited during depuration at 15°C. This study suggests that depuration at 15°C showed higher reductions of both *V. parahaemolyticus* and *V. vulnificus* because at this temperature the oyster pumped water faster and the growth of *Vibrio* was limited. Depuration conducted at 10°C had no advantage over the process conducted at 22°C probably due to the low water-pumping activity of the Gulf oyster at 10°C. Depuration at 5°C resulted in little or no reduction of *V. parahaemolyticus* or *V. vulnificus* suggesting

that the Gulf oyster stops its water-pump activity at this water temperature. This result is similar to a previous report showing approximately 1-log reduction of *V. vulnificus* in shellstock oyster after 14 days of storage at 4°C (Kasper and Tamplin 1993). The effects of low-temperature depuration for reducing *V. parahaemolyticus* or *V. vulnificus* in the Gulf oysters were further studied by depurating inoculated oyster in ASW at 15°C for 96 h. Results showed that the reductions of *V. parahaemolyticus* and *V. vulnificus* in the Gulf oyster increased from 2.1 and 2.9 log MPN/g after 48 h of ASW depuration to 2.6 and 3.3 log MPN/g, respectively, after 96 h of treatments (Figure 2.4). These results further demonstrated that ASW depuration at 15°C could effectively reduce both *V. parahaemolyticus* and *V. vulnificus* in the Gulf oyster. Both *V. parahaemolyticus* and *V. vulnificus* in the oyster could be reduced by >2.5 log MPN/g after 96 h of depuration at 15°C.

It is not clear why the low-temperature ASW depuration was more effective in reducing *V. vulnificus* than *V. parahaemolyticus* in the Gulf oyster. However, pure cultures of *V. parahaemolyticus* have been reported to be more resistant than pure cultures of *V. vulnificus* to high-pressure processing (Cook 2003) and EO water's bactericidal activity (Ren and Su 2006). A pressure treatment of 200 MPa for 131 s was adequate to achieve a 5-log reduction of *V. vulnificus* in phosphate-buffered saline while a same treatment for 427 s was required to achieve the same degree of reduction of *V. parahaemolyticus* in the same medium.

2.5 CONCLUSION

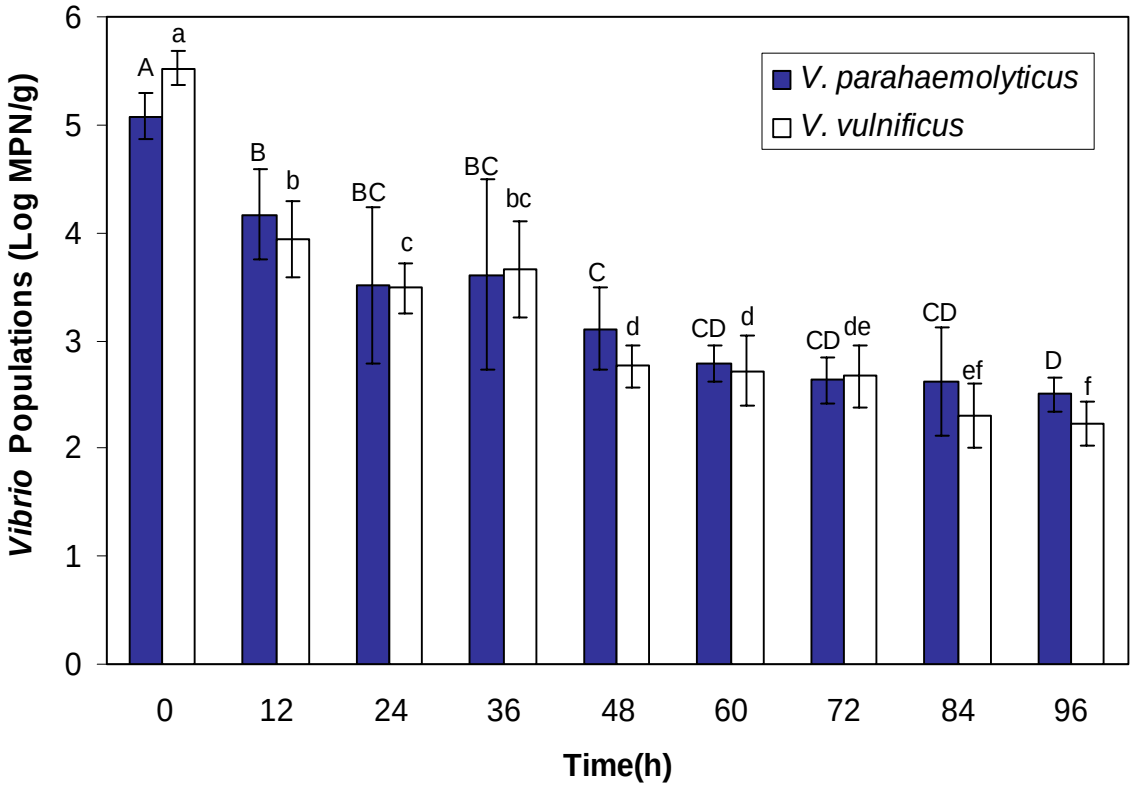

Figure 2.4 Survival of *V. parahaemolyticus* (solid bars) and *V. vulnificus* (hollow bars) in laboratory-inoculated oysters depurated in artificial seawater (ASW) at 15°C for 96 h. Data are means of four determinations ± standard deviations. Means with the same letter are not significantly different (P>0.05).

Depuration of oyster in EO water at various temperatures had no apparent advantages over ASW depuration in reducing *V. parahaemolyticus* or *V. vulnificus* in the Gulf oyster despite of the strong antimicrobial activity of EO water. Reducing ASW depuration temperature to 15°C could enhance the reductions of *V. parahaemolyticus* and *V. vulnificus* in oyster. Depuration at 15°C for 96 h could be used as a post-harvest treatment to reduce *V. parahaemolyticus* and *V. vulnificus* in the Gulf oyster by more than 2.5 log MPN/g.

2.6 ACKNOWLEDGEMENT

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Chapter III

Effects of Frozen Storage on Inactivating *Vibrio parahaemolyticus* in Pacific Raw Oysters (*Crassostrea gigas*)

3.1 ABSTRACT

Freezing and frozen storage is commonly used to prevent bacterial growth in food. This study investigated effects of frozen storage at various temperatures (-10, -23 and -30°C) on *V. parahaemolyticus* in half-shell Pacific raw oysters. Raw Pacific oysters were inoculated with five-strain cocktail of *V. parahaemolyticus* at a level of approximately 3.5×10^5 MPN/g. Inoculated oysters were subjected to an ultra-low, quick freezing process and stored at -10, -23 and -30°C for 4 months. The densities of *V. parahaemolyticus* in Pacific oyster decreased faster when stored at -10 than at -23 or -30°C. Populations of *V. parahaemolyticus* in oysters were reduced by 2.45, 1.71, and 1.45 log MPN/g in oysters after one month of storage at -10, -23, and -30°C, respectively. The reductions increased to 3.82 (-10°C), 3.14 (-23°C), and 2.28 (-30°C) log MPN/g after four months of storage. Holding half-shell Pacific oyster at -10°C for three months or at -23°C for four months was capable of achieving a greater than 3-log (MPN/g) reduction of *V. parahaemolyticus* in inoculated oysters.

Key words: *Vibrio*, *Vibrio parahaemolyticus*, storage temperature, freezing, oysters

3.2 INTRODUCTION

Vibrio parahaemolyticus is a well-documented causative agent of acute human gastroenteritis associated with ingestion of raw or undercooked shellfish and is recognized as a major cause of diarrhea associated with seafood consumption in the United States (Ayres 1978; Dalsgaard 1998; Liston 1990). The Centers for Disease Control and Prevention (CDC) estimated that each year there are approximately 2,800 cases of *V. parahaemolyticus* illness associated with the consumption of raw oysters (CDC 2006).

Several outbreaks of *V. parahaemolyticus* infection associated with raw oyster consumption have been documented in the U.S. since 1997. Between 1997 and 1998, four major outbreaks of *V. parahaemolyticus* infections involving more than 700 cases of illness associated with consumption of raw oysters occurred in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions of the U.S. (CDC 1998, 1999). In the summer of 2004, 14 passengers on board a cruise ship in Alaska developed gastroenteritis after eating raw oysters produced in Alaska (McLaughlin and others 2005). In the summer of 2006, *V. parahaemolyticus* caused another outbreak linked to raw oyster consumption. More than 100 people in Oregon, Washington, and British Columbia of Canada were sicken by *V. parahaemolyticus* after eating raw oysters harvested from the Puget Sound in Washington and British Columbia.

Additional outbreaks (74 cases) have also been reported in New York City restaurants and stores associated with eating raw oysters from the Pacific Northwest. Between May 20 and July 31 of 2006, a total of 177 cases of illness (three hospitalized and no fatalities) were reported in New York City and States of New York, Oregon, and

Washington (CDC 2006). The number of confirmed cases from the outbreak was greater than the average number reported between May and July from 2000 to 2004 in the entire U.S. Immediately following the outbreak, the U.S. Food and Drug Administration (FDA) advised consumers not to consume raw oysters harvested in the Pacific Northwest and forbad sale of oysters from the infected area for raw consumption until the threat was over (USFDA 2006). The often perceived and occasionally very real threat of *V. parahaemolyticus* infection following consumption of raw or undercooked oysters is a major concern for public health and causes substantial economic losses to the shellfish industry.

To limit growth of *V. parahaemolyticus* in raw oysters, the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish established time-to-temperature regulations that limit the maximum time of exposure of oysters to elevated temperatures. Shellfish harvested for raw consumption needs to be cooled down to 10°C (50°F) within 10, 12, and 36 h of harvest when the average monthly maximum air temperature is ≥27°C (81°F), between 19 and 27°C (66-80°F), and <18°C (66°F), respectively (NSSP 2003). However, exposure of products to elevated temperatures during retail storage cannot be totally avoided.

Freezing is a commonly used method to preserve product quality by inhibiting growth of bacteria. Certain oyster producers have utilized the technology to deliver high quality frozen oysters to consumers for raw consumption. Frozen storage has been reported capable of achieving reductions of *V. parahaemolyticus* in oyster meat. Muntada-Garriga and others (1995) reported that viable cells of *V. parahaemolyticus* (10^{5-7} CFU/g) in oyster homogenates were completely inactivated by freezing at -18 and -

24°C for 15 to 28 weeks depending on initial populations of the microorganism and freezing temperatures. A long-term storage (4-6 months) of half shell Gulf oyster at -20°C was reported capable of reducing low levels of *V. vulnificus* (<1,000 cells per gram of oyster) in oyster to non-detectable levels (Andrews 2004). However, no studies have been conducted to determine effects of frozen storage on reducing *V. parahaemolyticus* in the Pacific oyster. This study was conducted to determine effects of an ultra-low (liquid nitrogen) freezing process followed by frozen storage (-10, -23, and -30°C) on inactivating *V. parahaemolyticus* in half shell Pacific raw oysters.

3.3 MATERIALS AND METHODS

3.3.1 *Vibrio parahaemolyticus* cultures. Five clinical strains of *V. parahaemolyticus* 10290 (serotype O4:K12), 10292 (serotype O6:K18), 10293 (serotype O1:K56), BE98-2029 (serotype O3:K6) and 027-1C1 (serotype O5:K15) obtained from the U.S. Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, WA, USA) were used in this study. Each culture was individually grown in tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 1.5% NaCl (overall 2% NaCl) at 37°C overnight (12-16 h). The overnight culture was transferred to a fresh TSB containing 2% salt and incubated at 37°C for 4 h. The enriched cultures were pooled into a sterile centrifuge tube and harvested by centrifugation at 3,000 × g (Sorvall RC-5B, Kendro Laboratory Products, Newtown, CT, USA) at 5°C for 15 min. Pelleted cells were re-suspended in sterile 2% NaCl solution to produce a multi-strain cocktail suspension of approximately 10^9 CFU/ml.

3.3.2 Oyster preparation. Raw Pacific oysters were obtained from a local shellfish farm in Washington and shipped to the laboratory in a cooler with ice gel upon harvest. The oysters were briefly washed with tap water to remove mud on shell and placed in circulating artificial seawater (ASW, salinity: 29.6 ppt), prepared by dissolving Instant Ocean Salts (Aquatic Eco-System, Inc., Apopka, FL) in deionized water, at room temperature for 3-4 h. The oysters were then inoculated with *V. parahaemolyticus* by transferring the oysters to a tank of ASW containing the *V. parahaemolyticus* cocktail of approximately 10^5 CFU/ml. The inoculation was conducted at room temperatures overnight (12-14 h) with water being circulated at a rate of 10 L/h and air being pumped into the tank to facilitate colonization of *V. parahaemolyticus* in oysters (approximately 3.5×10^5 MPN/g).

3.3.3 Effects of ultra-low freezing and frozen storage on *V. parahaemolyticus* in oysters. Oysters inoculated with *V. parahaemolyticus* were packed in coolers with ice gels immediately after inoculation and delivered to the shellfish farm within 3 h. The top shell of each oyster were removed by skilled workers and the half-shell oysters were sent through a liquid nitrogen gas tunnel [-140°F (-95.5°C) for 12 min] for quick-freezing and glazed with tap water using a spray faucet. Frozen oysters were packed in cardboard boxes and brought back to the laboratory within 3 h and stored at –10, -23, and –30°C freezers. Populations of *V. parahaemolyticus* in oysters were determined before and after the freezing process and once a month for up to 4 months.

3.3.4 Microbiological analysis. *V. parahaemolyticus* in oysters was determined with the 3-tube most probable number (MPN) procedure described in FDA's Bacteriological

Analytic Manual (USFDA 1998). At each testing time, 5 oysters from each storage temperature were randomly removed from the freezers and let thaw in a refrigerator (5-7°C) overnight. Each oyster meat was placed in a sterile blender jar and blended with 9 volumes of sterile alkaline peptone water (APW) at high speed for 1 min to prepare a 1:10 sample suspension. Two additional ten-fold dilutions of each oyster sample were prepared with sterile APW. All sample dilutions were individually inoculated into three tubes of alkaline peptone salt broth (APS). Inoculated APS tubes were incubated at 35-37°C for 16-18 h. Each enriched APS showing turbidity after incubation was streaked onto individual thiosulfate-citrate-bile salts-sucrose agar (TCBS) and incubated at 35-37°C for 18-24 h. Formation of colonies that are round (2-3 mm diameter) and green or bluish on TCBS was considered positive for *V. parahaemolyticus*. Total populations of *V. parahaemolyticus* in oysters were determined by converting numbers of APS tubes that were positive for *V. parahaemolyticus* to MPN/g using a MPN table. Results were reported as means of five determinations.

3.3.5 Data Analysis. Results of microbiological tests were transformed into log values for statistical analyses. Populations of *V. parahaemolyticus* in oysters at different times within a treatment or between treatments were analyzed with Tukey-Kramer multiple comparison test (S-Plus, Insightful Corp., Seattle, WA). Significant differences between means of treatments were established at a level of P=0.05.

3.4 RESULTS AND DISCUSSION

V. parahaemolyticus is one of the bacteria with a fast generation time (Natarajan and others 1980). Therefore, a low level of *V. parahaemolyticus* in raw seafood could

render public health hazard if the temperature of storage is not properly controlled. As expected, the density of *V. parahaemolyticus* in inoculated oyster remained at a similar level after the ultra-low quick-freezing process (Figure 1). However, subsequent storage of oysters at -10, -23, and -30°C resulted in considerable decreases in *V. parahaemolyticus* counts. The populations of *V. parahaemolyticus* in oysters declined much faster during the first month of frozen storage at all three temperatures and continued to decline at a slower rate during the rest of storage. The decline trends were similar for all three temperatures although the slopes varied. The densities of *V. parahaemolyticus* in oysters decreased by 2.45, 1.71, and 1.45 log MPN/g in oysters after one month of storage at -10, -23, and -30°C, respectively (Figure 1). The decreases increased to 3.82 (-10°C), 3.14 (-23°C), and 2.28 (-30°C) log MPN/g after four months of storage.

The rapid decline of *V. parahaemolyticus* in oyster observed at early post-freezing storage was similar to previous studies of storing oyster meat homogenate inoculated with *V. parahaemolyticus* at freezing temperatures (Muntada-Garriga and others 1995). Johnson and Liston (1973) also reported that freezing shucked oyster at -15 and -30°C was capable of reducing viable *V. parahaemolyticus* (10^{5-7} log MPN/g) cells by 1 to 2 log units within 20 days.

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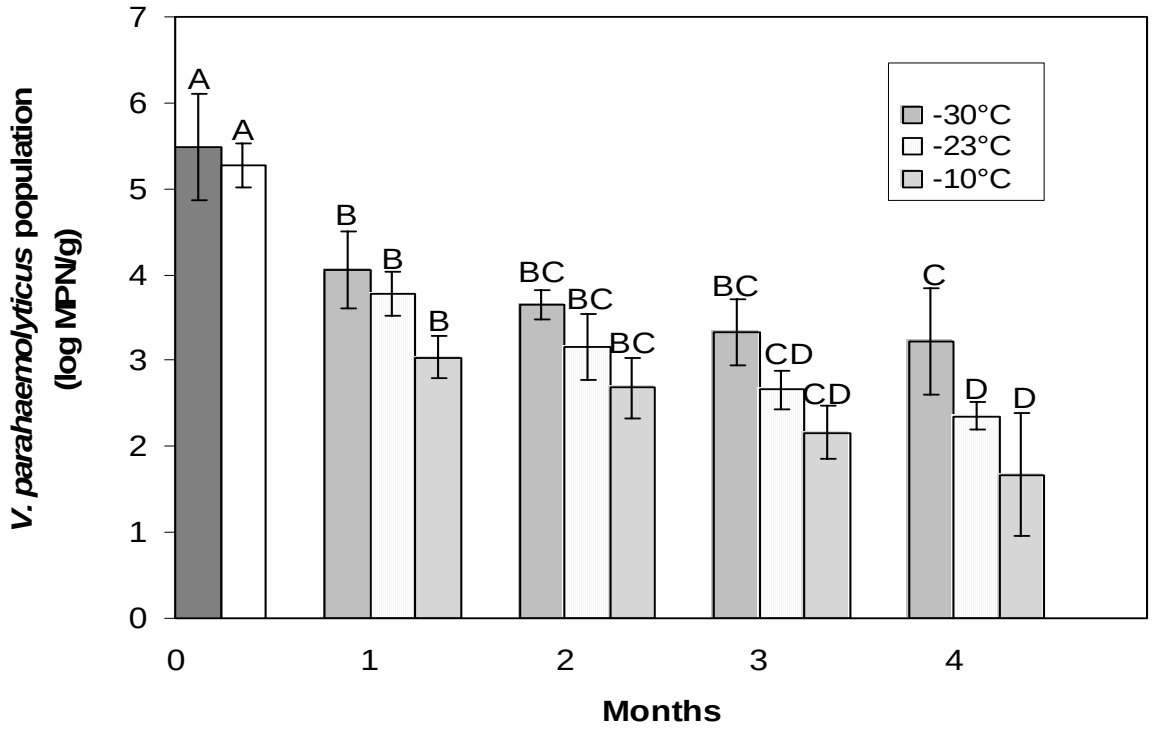

Figure 3.1 Effect of storage temperatures (-10, -23, and -30°C) on reducing *V. parahaemolyticus* in half-shell oysters. Solid bar: initial population before freezing (5.49 log MPN/g). Hollow bar: population after freezing (5.27 log MPN/g). Data are means of five determinations ± standard deviations. Means with the same letter are not significantly different (P>0.05).

units within 20 days.

While the populations of *V. parahaemolyticus* in oysters all decreased when stored at -10, -23, and -30°C, the bacterium appeared to survive better in oysters at -23 or -30°C than at -10°C. This might be mainly because of intracellular ice crystals formed in the bacteria at lower freezing temperatures (-20 or -30°C) are smaller than those formed at a higher temperature such as -10°C. Therefore, bacteria survive better at lower freezing temperatures due to less cell damage (Jay and others 2005). Boutin and others (1985) studied the survival of several strains of *V. parahaemolyticus* in shrimp homogenates at -20 and -80°C and found the counts remained unchanged at -80°C after initial 1-log reduction of the cells in sample after a freezing and thawing process. However, 5-log reductions in the *V. parahaemolyticus* populations were observed in the shrimp homogenates after 25 days of storage at -20°C. Johnson and Liston (1973) also reported that mortality rate of *V. parahaemolyticus* in shucked oysters stored at -15°C is higher than that of oysters stored at -30°C.

This study showed that storing half-shell oyster at -23°C for four months following an ultra-low quick freezing process achieved a greater than 3-log (3.14 log MPN/g) reduction of *V. parahaemolyticus*. A greater reduction (3.82 log MPN/g) of *V. parahaemolyticus* was observed in oysters stored at -10°C. Storing oysters at -10°C has advantages over storing oysters at -23 or -30°C because of less energy usage and greater reduction of *V. parahaemolyticus* in oyster. However, oyster stored at -23 or -30°C for a long term might have a better organoleptic quality and consumer acceptance than those stored at -10°C because larger ice crystal is expected to form in oysters stored at -10°C than at -23 or -30°C.. Therefore, sensory study needs to be conducted to compare changes

of sensory characteristics and consumer acceptance of oysters stored at -10°C with those of oysters stored at -23°C.

3.5 CONCLUSIONS

Frozen storage can be used as a post-harvest process for reducing *V. parahaemolyticus* contamination in raw Pacific oyster. The densities of *V. parahaemolyticus* in Pacific oyster stored at -10, -23, and -30°C all significantly decreased in the study with the greatest reduction being observed at -10°C followed by -23 and -30°C. Holding half-shell Pacific oyster at -10°C for three months or at -23°C for four months was capable of achieving a greater than 3-log (MPN/g) reduction of *V. parahaemolyticus* in inoculated oysters.

3.6 ACKNOWLEDGEMENT

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Chapter IV

Conclusion and Future Study

Contamination of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oysters is a human health concern because oysters are commonly consumed raw. Depuration of oyster in a solution exhibiting strong antimicrobial activities such as electrolyzed oxidizing (EO) water was found ineffective in reducing cells of *V. parahaemolyticus* or *V. vulnificus*. Reductions of *V. parahaemolyticus* and *V. vulnificus* in oyster by EO water depuration at 22°C for 48 h was limited to 0.7 and 1.4 log MPN/g, respectively. Depuration with EO water at lower temperature (5-15°C) did not enhance reductions of both *Vibrio* species in the oysters. It is obvious that the acidic and chlorine-containing EO water creates an unfavorable growth condition for oyster stopping the water filtering activity after exposure to EO water despite of changes in water temperatures.

Depuration of oysters in artificial seawater (ASW) appeared to be more effective than EO water in reducing *Vibrio* contamination in oysters. Populations of *V. parahaemolyticus* and *V. vulnificus* in the Gulf oysters were slightly reduced by 1.2 and 2.0 log MPN/g after 48 h of ASW depuration at 22C°. This confirms that depuration at room temperature is not very effective in reducing *V. parahaemolyticus* or *V. vulnificus* in oysters. However, ASW depuration at a lower temperature (15°C) for 48 h significantly increased reductions of *V. parahaemolyticus* and *V. vulnificus* in the Gulf oysters to 2.1 and 2.9 log MPN/g, respectively. However, depuration of oyster in ASW at 10 and 5°C were found less effective in reducing the *Vibrio* spp. in oyster due to low

water-pumping activity of oyster at such temperatures. This study demonstrated that ASW depuration at 15°C might not affect oyster's normal pumping rate and also retard multiplication of *Vibrio* in oysters. An extended depuration with ASW at 15°C for 96 h was found capable of achieving 2.6 and 3.3 log MPN/g of reductions of *V. parahaemolyticus* and *V. vulnificus*, respectively, in the Gulf oyster. This depuration process was the first post-harvest process that could achieve greater reductions of both *V. parahaemolyticus* and *V. vulnificus* in oyster without adverse effects on the oysters.

This study found that low-temperature acidic electrolyzed oxidizing (EO) water depuration had no impacts on reducing *Vibrio parahaemolyticus* or *Vibrio vulnificus* in oysters. However, depuration with artificial seawater at 15°C greatly reduced the levels of both *Vibrio* species in contaminated Gulf oysters. The high acidity of the EO water is believed to be the main factor that the EO water depuration was not effective in reducing *Vibrio* contamination in oyster because oyster tends to stop its pumping activity under such an acidic environment.

A recent development in the EO water technology allows the production of neutral electrolyzed water (NEW) with near neutral pH. The antimicrobial activity of NEW has been reported to be effective in inactivating bacteria like acidic EO water. The neutral pH of NEW might allow oyster to exhibit normal water-filtering activity. Depuration of oyster in NEW may be a future study of potential application of electrolyzed water for reducing *Vibrio* contamination in oyster post harvest. Sensory studies should be conducted to determine the organoleptic quality and consumer acceptability of electrolyzed water processed oysters if the NEW depuration was found effective in reducing *Vibrio* species in oysters.

In addition to ASW depuration at 15°C, frozen storage at -10, -23 and -30°C could also be used as a means to reduce *V. parahaemolyticus* and *V. vulnificus* contamination in oysters. Populations of *V. parahaemolyticus* in half-shell Pacific oysters were reduced by 2.45, 1.71, and 1.45 log MPN/g after one month of storage at -10, -23, and -30°C, respectively. The reductions increased to 3.82 (-10°C), 3.14 (-23°C), and 2.28 (-30°C) log MPN/g after four months of storage. Holding half-shell Pacific oyster at -10°C for three months or at -23°C for four months was capable of achieving a greater than 3-log (MPN/g) reduction of *V. parahaemolyticus* in the oysters.

Holding half-shell oyster at -23°C for a few months is used by several shellfish producers to reserve products for future shipments. This study showed that the practice could also reduce *V. parahaemolyticus* contamination in oysters and the reductions of the organism would be greater if oysters are stored at -10°C rather than at-23°C. Further studies are needed to evaluate potential changes in sensory characteristics and consumer acceptance of oysters stored at -10°C.

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