AN ABSTRACT OF THE THESIS OF

Tingting Ren for the degree of Master of Science in Food Science and Technology presented on December 8, 2005.
Title: Electrolyzed Oxidizing Water Treatment for Reducing *Vibrio* Contamination in Oysters

Redacted for privacy

Abstract approved: _____
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Oysters can be easily contaminated with spoilage and pathogenic bacteria through contaminated waster. The United States produces more than 27 million pounds of oysters each year and most of them are sold and consumed raw without further processing. Growth of naturally contaminating bacteria in oysters during storage results in loss of quality and product value. Contamination of pathogenic bacteria such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oysters is also a safety concern of the industry and consumers.

The traditional depuration process for reducing bacterial contamination in oysters using clean seawater has been reported to be ineffective in reducing *Vibrio* contamination in oysters. This study investigated effects of electrolyzed oxidizing (EO) water treatment on reducing both *V. parahaemolyticus* and *V. vulnificus* contamination in oysters.

Freshly harvested Pacific oysters were inoculated with 5-strain cocktail of *V. parahaemolyticus* or *V. vulnificus* at levels of 10^4 and 10^6 MPN/g. Inoculated oysters were held in EO water (chlorine: 30 ppm, pH: 2.82, ORP: 1131 mV) containing 1% NaCl at room temperature. Reductions of *V. parahaemolyticus* and *V. vulnificus* in oysters during the treatment were determined at 0, 2, 4, 6, and 8 h.

EO water containing 30 ppm of chlorine was found to exhibit strong bactericidal effects against pure cultures of *V. parahaemolyticus* and *V. vulnificus* (>6.6-log reductions in 15s). Holding oysters inoculated with *V. parahaemolyticus* or *V. vulnificus* in EO water (30 pm chlorine) containing 1% NaCl for 4-6 h resulted in significant reductions of *V. parahaemolyticus* and *V. vulnificus* by 1.13 and 1.05 log MPN/g, respectively. However, extended exposure (>12 h) of oysters in EO water containing high levels of chlorine (>30 ppm) was found to be detrimental to oysters.

EO water could be used as a post-harvest treatment to reduce *Vibrio* contamination in oysters. However, treatment should be limited to 4-6 h to avoid death of oysters. Further studies are needed to determine effects of EO water treatments on sensory characteristics of treated oysters.

Electrolyzed Oxidizing Water Treatment for Reducing *Vibrio* Contamination in Oysters

by
Tingting Ren

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented December 8, 2005
Commencement June 2006

Master of Science thesis of Tingting Ren presented on December 8, 2005.

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Tingting Ren, Author

Acknowledgements

Life is a journey to be savored each step of the way. The journey can be happy and exciting, also it can be tough and at times, challenging. During the two years of pursuing my master's degree in the U.S., I increased my knowledge and skills in food science. Also I have gained valuable experiences, which would definitely be helpful for my future growth. Also, there are many people I would like to express my sincere thanks for their helps.

I would like to say "thank you very much" to my major advisor, Dr. Yi-Cheng Su, for providing the opportunity of furthering my study in the U.S., and for his great guidance through my Master years. His inspiration, patience and clear explanations enabled me to learn scientific knowledge and experimental methods step by step.

Many thanks to people in the OSU Seafood Lab and Food Science Department, who gave me a lot of helps and provided such a stimulating environment. I learned and grew up here, and made new friends all over the world with different cultures. Special thanks go to my colleague, Jingyun, for helping with my research and teaching me a lot about lab techniques.

Finally, I am very grateful to my parents who born me, raised me, loved and supported me with heart and soul. It is to them this work is dedicated.

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CHAPTER 1

Introduction

Foodborne illness can be extremely costly to the food industry and the nation's economy. The estimated yearly loss from foodborne diseases in the U.S. ranges from 5 to 6 billions in direct medical expenses and lost of productivity. *Vibrio parahaemolyticus* and *Vibrio vulnificus* are human pathogens that occur naturally in estuarine environments and are commonly found in shellfish, particularly raw oysters. Consumption of raw oysters contaminated with these pathogens can lead to development of acute gastroenteritis or septicemia through consumption of contaminated shellfish (FDA 2001).

Oysters are filter-feeding organisms and can easily be contaminated with spoilage and pathogenic bacteria through contaminated water in the growing environments. The United States produces more than 27 million pounds of oysters each year (Hardesty 2001) and most of them are sold live or shucked without further processing. Accumulation of pathogenic bacteria such as *V. parahaemolyticus* and *V. vulnificus* in oysters is a safety concern of the industry and consumers. It is estimated that 20 million Americans eat raw shellfish, making raw or undercooked shellfish the

biggest seafood hazard.

V. parahaemolyticus is the leading cause of bacterial diarrhea associated with seafood consumption in the U.S. and *V. vulnificus* could cause septicemia with 50% mortality (Andrews 2004). Several outbreaks of *V. parahaemolyticus* infections involving more than 700 cases of illness associated with consumption of raw oysters occurred between 1997 and 1998 in the United States (CDC 1998, 1999). A recent national survey of 370 lots of oysters sampled from restaurants, oyster bars, retail and wholesale seafood markets throughout the U.S. showed that the densities of *V. parahaemolyticus* in market oysters were as high as 1,000 MPN/g in the summer (Cook and others 2002). The latest outbreak occurred in summer of 2004 on board of a cruise ship in Alaska (McLaughlin and others 2005) indicates that the association of these pathogens with raw shellfish continue to be a safety problem for the shellfish industries. Development of an effective post-harvest treatment to reduce *Vibrio* contamination in oysters is needed for reducing the health hazards associated with raw oyster consumption.

Several methods, including high pressure processing (Calik and others 2002; He and others 2002), irradiation processing (Kilgen and other 1998; Andrews and others 2002), freezing (Hesselman and others 1999), low temperature pasteurization (Andrews and others 2000), and chemical processes (Borazjani and others 2003),

have been developed as post-harvest treatments for reducing *Vibrio* contamination in oysters. However, those treatments may cause changes in flavor and texture after the treatment and oysters are often killed during the processes. Development of a process to reduce the contamination without killing oysters remains a great need of the industry.

Depuration is a controlled purification process allowing shellfish to purge sand and grit from the gut into 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). Unfortunately, traditional depuration process with clean seawater was ineffective in reducing *Vibrio* contamination in oysters due to the colonization of *Vibrio* cells in the digestive tract. Studies have shown no significant reduction of naturally occurring *V. parahaemolyticus* in oysters after the depuration process (Eyles and Davey 1984), and it required 16 days to depurate laboratory-contaminated *V. vulnificus* in oysters to non-detectable level (Kelly and Dinuzzo 1985). However, replacing clean seawater with solutions containing strong anti-bacterial activities might improve the efficacy of depuration process for reducing *Vibrio* spp. in contaminated oysters.

Electrolyzed water, generated with electrolysis of a dilute salt colution (0.1-0.5% NaCl), has been reported to have strong anti-microbial activities against

many pathogens including *Salmonella enteritidis*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 (Venkitanarayanan and others 1999a; Kim and others 2000; Park and others 2002a). Two types of water are produced from the water electrolysis with either high reducing (alkaline water) or high oxidizing (acidic water) potential. The electrolyzed oxidizing (EO) water has a pH of about 2.5 with strong bactericidal activities and has been reported capable of reducing bacterial contamination on fresh-cut vegetables, shell eggs, and cutting boards (Izumi 1999; Venkitanarayanan and others 1999b, Koseki and Itoh 2001; Park and others 2005). In addition, EO water could be used as a sanitizer to effectively reduce bacterial contamination on food-processing surfaces (Park and others 2002b, Liu and others 2005). It might be used in oyster depuration process to effectively reduce *V. parahaemolyticus* and *V. vulnificus* cells in oysters. A major advantage of utilization of EO water as a sanitizer is that it is produced with water and sodium chloride without other added chemicals. Therefore, it is easy to use and has less adverse impact to the environments (Hsu 2003).

The objectives of this study were to (1) determine the bactericidal effects of EO water against *V. parahaemolyticus* and *V. vulnificus*, (2) identify the optimal EO water for reducing *V. parahaemolyticus* and *V. vulnificus* in oysters, and (3) evaluate potential application of EO water as a post-harvest process for reducing *V.*

parahaemolyticus and *V. vulnificus* in oysters. Freshly harvested Pacific oysters were inoculated with 5-strain mixture of *V. parahaemolyticus* or *V. vulnificus*. Survivals of *Vibrio* cells in laboratory-contaminated oysters were determined with different types of EO water generated with an EO water generator (Model V-500, Electric Aquagenics Unlimited, Inc. Lindon, UT).

CHAPTER 2

LITERATURE REVIEW

Tingting Ren

Vibrio Species

Members of the genus *Vibrio* are natural habitants of the marine environments and are gram-negative, halophilic, asporogenous rods that are either straight or a single and rigid curve. Most of them have a single polar flagellum and are motile when grown in liquid medium. Three species, *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* are well-documented human pathogens and have been linked to foodborne illnesses associated with seafood consumption, particularly raw or lightly cooked shellfish (Sakazaki and others 1963; Oliver 1989; McPherson and others 1991; Rippey 1994; Kaper and others 1995; McLaughlin 1995). Other species, such as *V. mimicus*, *V. alginolyticus* and *V. hollisae*, can also cause illness in human and are occasional human pathogens (Hickman 1982; Abbott 1994; Ji 1989).

Most *Vibrio* species produce oxidase and can ferment glucose without gas production (Baumann and Schubert 1984). Table 2.1 lists the biochemical characteristics of human pathogenic *Vibrionaceae* commonly encountered in seafood (FDA 2004). Both *V. alginolyticus* and *V. cholerae* can ferment sucrose, while *V. vulnificus* can ferment lactose.

Table 2.1 Biochemical characteristics of human pathogenic *Vibrionaceae* commonly encountered in seafood (Data adopted from FDA 2004)

		V. alginolyticus	*V. cholerae*	*V. hollisae*	*V. Mimicus*	*V. parahae-molyticus*	*V. vulnificus*
TCBS agar		Y	Y	NG	G	G	G
mCPC agar		NG	P	NG	NG	NG	Y
Oxidase		+	+	+	+	+	+
Growth in (w/v):	0% NaCl	-	+	-	+	-	-
	3% NaCl	+	+	+	+	+	+
	6% NaCl	+	-	+	-	+	+
	8% NaCl	+	-	-	-	+	-
	10% Nacl	+	-	-	-	-	-
Growth at 42 °C		+	+	nd	+	+	+
Acid from:	Sucrose	+	+	-	-	-	-
	D-Cellobiose	-	-	-	-	V	+
	Lactose	-	-	-	-	-	+

Abbreviations: TCBS, thiusulfate-citrated-bile salts-sucrose; mCPC, modified cellobiose-polymyxin B-colistin;
Y= yellow NG= no or poor growth nd=not done
G= green V=variable among strains P=purple

Table 2.2 Clinical syndromes associated with Florida *Vibrio* infections, 1981-1993 (Data adopted from Hlady and Klontz 1993)

Vibrio species	Gastroenteritis	Primary septicemia	Wound infection	other	unknown	Total
V. parahaemolyticus	120	16	56	1*	13	206
V. vulnificus	16	75	47		3	141
V. cholerae non-O1	87	20	11	4†	8	130
V. hollisae	50	3	4		2	59
V. alginolyticus	6		37	6‡	3	52
V. fluvialis	35	3	5	2ζ	3	48
V. mimicus	34	1	1	1*	3	40
V. cholerae O1	7					7

* Urinary tract infection

† 2 pulmonary infections of drowing victims, 1 ear infection, 1 urinary tract infection.

‡ 2 pulmonary infections of drowing victims, 2 ear infection, 2 urinary tract infection.

ζ Gall bladder infections

Vibrio parahaemolyticus

V. parahaemolyticus can grow in the presence of 1-8% NaCl with optimal levels between 2% and 4% (Sakazaki 1979). Optimal temperatures for *V. parahaemolyticus* to grow are between 35 and 39 °C with an upper limit of 44 °C (Jackson 1974). 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. parahaemolyticus* remian viable, but will not produce visible colonies on the growth media.

V. parahaemolyticus is frequently isolated from coastal waters and seafood in temperate zones. It accounts for numbers of gastroenteritis outbreaks in the U.S. related to oyster consumption (Nolan and others 1984; Rippey 1994; Kaysner 2000). Occurrence of *V. parahaemolyticus* in the marine environment is known to relate to the temperature of water. It is more frequently found in the marine environment when water temperature exceeds 20 °C (Jay and others 2005). In temperate area, a seasonal occurrence of *V. parahaemolyticus* in shellfish has been reported in the warmer months of the year. However, the organism can be found in the environment and in shellfish around the year in subtropical area such as in Florida.

V. parahaemolyticus is the most frequent cause of foodborne gastroenteritis associated with seafood consumption in the U.S. (Daniels and others 2000). Typical

symptoms were diarrhea, abdominal pain, nausea, vomiting, headache, fever and chills. Occasionally, infection could also cause would infection and septicemia. The organism was identified as a foodborne pathogen in 1951 after being involved in a food poisoning outbreak in Japan (Fujino and others 1974). While *V. parahaemolyticus* is the leading cause of diarrhea associated with seafood consumption, most strains of *V. parahaemolyticus* are non-pathogenic to human. Pathogenic strains of *V. parahaemolyticus* are characterized as being capable of producing thermostable direct hemolysin (TDH) and/or thermostable related hemolysin (TRH). The thermostable direct hemolysin (TDH) is a cardiotrophic, cytotoxic protein with a molecular weight of 42,000 daltons. The TDH acts on cellular membranes as a pore-forming toxin that changes ion flux in intestinal cells, thus resulting in a secretory response and diarrhea (Raimondi and others 2000). It can be identified by its ability to produce β-haemolysis on a special blood agar (Wagatsuma agar) known as Kanagawa phenomenon (KP) (Okuda and Nishibuchi 1998).

Despite epidemiological investigations revealed a strong tie between the Kanagawa phenomenon and pathogenicity of *V. parahaemolyticus*, KP-negative strains had been isolated from outbreak patients (Honda and others 1987, 1988). The isolate did not carry the gene encoding TDH, but produced a TDH-related hemolysin

(TRH). The TRH was reported to be immunologically similar but physiochemically different to TDH. The gene (*trh*) encoding the TRH has been sequenced and found to relate to clinical strains of *V. parahaemolyticus* (Kishishita and others 1992). A survey of 285 strains of *V. parahaemolyticus* revealed that the *trh*-positive strains had strong association with gastroenteritis (Shirai and others 1990). Analysis of sequences of genes encoding TRH (*trh*) and TDH (*tdh*) revealed approximately 70% identity of nucleotide sequence (Kishishita and others 1992). However, unlike TDH, TRH is labile to heat treatment at 60 °C for 10 min. The mechanism of TRH in causing infection seems to be similar to that of TDH. It induces Ca^{2+}-activated Cl^- channels that lead to altered ion flux. In addition, a thermolabile hemolysin (TLH) was found unique in all *V. parahaemolyticus* strains (Taniguchi and others 1986). Methods including PCR and gene probe assays have been developed to detect the *tlh*, *tdh* and *trh* genes in *V. parahaemolyticus*.

V. parahaemolyticus can be identified based on its serological antigens (O and K antigens) on cell surface. Currently, 13 O-group and 71 K-type antigens of *V. parahaemolyticus* have been identified and can be analyzed with commercially available antisea. Outbreaks of *V. parahaemolyticus* infections are usually caused by a variety of serotypes. However, increased incidences of gastroenteritis caused by *V. parahaemolyticus* serotype O3:K6 have been reported in many countries since 1996

(Chiou and others 2000; Vuddhakul and others 2000; Gonzáz-Escalona and others 2005; Martinez-Urtaza and others 2005). The first reported outbreak of *V. parahaemolyticus* O3:K6 infection in the U.S. occurred in Texas in 1998. A total of 416 persons developed gastroenteritis after eating raw oysters harvested from Galveston Bay. *V. parahaemolyticus* O3:K6 was isolated from stool specimens collected from 28 infected persons (Daniels and others 2000). The same clone was later involved in another outbreak related to shellfish consumption in Connecticut, New Jersey, and New York (CDC 1999). Occurrence of this highly virulent strain in the recent U.S. outbreaks has raised the concern about increased risks of *V. parahaemolyticus* infections from U.S. shellfish consumption.

Vibrio vulnificus

Vibrio vulnificus was first isolated by the US Centers for Disease Control and Prevention (CDC) in 1964 as a virulent strain of *V. parahaemolyticus*. In the 1970s, it was recognized as a distinct species of *Vibrio* and could 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).

Vibrio vulnificus can grow in the environment containing 0.5-6 % NaCl with an optimal temperatures for growth between 39-40 °C (FDA 2004). Cells of *V.*

vulnificus can be detected with modified cellobiose-polymyxin B-colistin (mCPC) agar. Growth of *V. vulnificus* on the mCPC agar results in formation of flat and yellow colonies. It has been reported that *V. vulnificus* could also enter the viable but non-culturable (VBNC) state when temperature dropped below 10 °C (Oliver 1995). Under the VBNC status, cells of *V. vulnificus* changed 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 an others 1997).

V. vulnificus is mainly found in seawater and seafoods, such as oysters and clams, in area with tropical and subtropical climates. In the U.S., *V. vulnificus* is more frequently found in the Gulf coast than in area with cooler water such as New England and the Pacific Northwest. Therefore, outbreaks of *V. vulnificus* infection mainly occurred in the Gulf coast and southeast regions due to warm climates. Typical syndromes of *V. vulnificus* infection include wound infections, primary septicemia, and gastroenteritis. This organism is responsible for 20 to 40 cases of primary septicemia each year in the U.S. with a 50% mortality rate among people with liver disease and high serum iron levels (Shapiro and other 1998).

V. vulnificus can be divided into two biotypes based on phenotypic and host differences. Strains of biotypes 1 are mainly associated with shellfish colonization

and human infection. Those strains produce indole and ornithine decarboxylase with the exhibition of several immunologically distinct lipopolysaccharide (LPS). Strains of biotype 2 appear to be related to marine vertebrates infections, especially in cultured eels, and can cause significant economic losses.

V. vulnificus may express an extracellular acidic polysaccharide capsule on its cell surface. Presence of this capsular polysaccharide (CPS) on cell surface of virulent strains of *V. vulnificus* was reported to be positively correlated with its level of virulence in the mouse model (Yoshida and others 1985; Wright and others 1990). In addition, *V. vulnificus* also exhibits various type of lipopolysaccharide (LPS) on cell surface. Both CPS and LPS are known potent mediators of bacterial septic shock through the induction of host pyrogenic responses (Strom and Paranjpye 2000). Although the CPS or LPS does not appear to be as pyrogenic as other bacterial endotoxins, presence of both CPS and LPS may produce a synergistic effect on inducing inflammation, tissue damage, and septicemic shock during systemic *V. vulnificus* infections. (Strom and Paranjpye 2000)

V. vulnificus can produce a number of degradative enzymes and toxins. Some of these extracellular proteins (ECPs) are important for this bacterium to survive in the estuarine environment. For example, chitinase produced by *V. vulnificus* may help its colonization and adhesion to the chitin exoskeletons of zooplankton. This

activity needs to be considered as a potential virulence factor that may lead to a rapid tissue destruction in susceptible hosts (Strom and Paranjpye 2000).

Vibrio cholerae

Vibrio cholerae can grow in the environment containing 0-3% NaCl with an optimal temperatures for growth between 35 and 37 °C. This organism can produce cholera toxin (CT) and cause a severe diarrheal disease known as cholera (Herrington and others 1988). The disease can be caused through consumption of seafoods contaminated with *V. cholerae*, but is mainly transmitted via contaminated water supplies. Direct person-to-person spread is not common. Outbreaks of cholera are reported all around the world and result in up to 120,000 deaths each year worldwide (World Health Organization 1995). Seven pandemics of cholera have been reported since the first one occurred in 1817 (Pollitzer 1959). The first six pandemics were all spread from the Indian subcontinent, usually the Ganges delta, to other countries and the seventh one was originated from the Sulawesi island of Indonesia (Kamal 1974).

Strains of *V. cholerae* can be classified into different serogroups based on its somatic (O) antigens. More than 150 (O) antigens have been identified for *V. cholerae* (Shimada and Sakazaki 1977; Shimada and others 1993; Shimada and

others 1994). These serotypes can be further divided according to the biochemical properties and susceptibility to bacteriophages. Most *V. cholerae* strains recovered from epidemic cholera contain a common somatic O1 antigen (Kaper and other 1995). However, a large cholera-like outbreak occurred in Bangladesh and India in 1993 was caused by *V. cholerae* non-O1 serogroup (Albert and others 1993). The strain was later designated O139 (Shimada and others 1993). Since then, the O1 and O139 antigens were considered the markers of epidemic potential (Kaper and others 1995). Strains of *V. cholerae* that did not produce agglutination with either anti-O1 or -O139 serum were referred as *V. cholerae* non-O1/O139 (Kaper and others 1995; Faruque and others 1998). The non-O1/O139 *V. cholerae* has been reported to cause sporadic cholera-like diarrheal disease, but was rarely involved in major outbreaks (Morris 1994; Sharma and others 1998).

Vibrio mimicus

Many strains of *V. cholerae* initially identified as atypical non-01 *V. cholerae* were renamed *V. mimicus* because of their similarities to *V. cholerae* (Davis and others 1981). *V. mimicus* has similar characteristics to *V. cholerae,* except the ability to ferment sucrose (Davis and others 1981; Shandera and others 1983; Spira and others 1984). Therefore, growth of *V. mimicus* on thiosulfate-citrate-bile salts-

sucrose (TCBS) agar results in formation of green colonies.

V. mimicus can grow in many common media containing 0-3% NaCl. Infection of *V. mimicus* has been linked to the consumption of raw or undercooked seafood (Rippey 1994). Between 1981 and 1993, 40 cases of *V. mimicus* infection were reported in Florida (Hlady and Klontz 1996). Typical syndromes of *V. mimicus* infection include diarrhea, nausea, vomiting, and abdominal cramps. Other syndromes such as fever, headache, and bloody diarrhea may also occur occasionally (Shandera and others 1983).

Vibrio infection associated with seafood consumption

Oyster is a filter-feeding animal and will accumulate Vibrio cells in the digestive tracts through filtering contaminated water. Human consumption of such contaminated oysters has caused numbers of diseases including diarrhea, typhoid fever, paratyphoid fever, cholera, etc (Wood 1976).

The first outbreak of *V. parahaemolyticus* infection in the U.S. was reported in 1971 involving consumption of contaminated crab meat (Molenda 1972). Since then, 42 outbreaks of *V. parahaemolyticus* infection have been documented (Yeung and Boor 2004). Among them, the largest outbreak occurred in the Pacific Northwest between July and August in 1997. A total of 209 persons suffered from

gastroenteritis after consumption of raw oysters harvested in California, Oregon, and Washington of the U.S. and in British Columbia of Canada (JAMA 1998). Table 2.2 lists the causative agents of 675 *Vibrio* infections occurred in Florida between 1981 and 1993 (Hlady and Klontz 1993). *V. parahaemolyticus* was found the leading cause of those infections followed by *V. vulnificus,* non-O1 *V. cholerae*, *V. hollisae*, *V. alginolyticus*, *V. fluvialis*, *V. mimicus*, and *V. cholerae* O1.

According to the Centers for Disease Control and Prevention (CDC), *V. parahaemolyticus* is responsible for about 5000 illnesses every year in the U.S. (Yeung and Boor 2004). A recent outbreak of *V. parahaemolyticus* gastroenteritis occurred on board of a cruise ship in Alaska in summer 2004 was linked to the consumption of Alaskan raw oysters (McLaughlin and others 2005). *V. parahaemolyticus* has never been detected in Alaskan oysters or marine environmental samples between 1995 and 2003 according to the Alaska Department of Environmental Conservation (DEC) (McLaughlin and others 2005). The unexpected outbreak occurred in Alaska indicated that *V. parahaemolyticus* infection associated with raw oyster consumption remained a safety concern.

Depuration

Depuration is a dynamic process that usually takes 48-72 h whereby shellfish, particularly oysters, are allowed to purge contaminants in tanks containing clean seawater (Fleet 1978). The process usually leads to a reduction of microbial contaminants in shellfish and therefore increases shelf life of refrigerated products. Studies have reported that shellfish moderately contaminated with most indicator bacteria (total bacterial count of about 10^5 cells/g) and pathogens such as *Escherichia coli*, *Bacillus cereus*, *Clostridium perfringens*, and *Campylobacter* species can be depurated to undetectable level in 48 h (Souness and Fleet 1991). In addition, contamination of toxin such as microcystin-LR and heavy metals such as mercury, manganese, cadmium, and selenium in oysters could also be reduced slowly through the deputation process (Denton and Burdon-Jones 1981; Burger and others 1998; Yokoyama and Park 2003). However, effectiveness of depuration on reducing bacterial contamination in oysters depends on several parameters including temperature, salinity, dissolved oxygen, and turbidity of water.

System Parameters

Water temperature

Maintaining an optimal water temperature is essential for the depuration process. Each shellfish species will pump at different ranges of temperature and shellfish grown at cold water tend to have a lower optimal depuration temperature than those grown at warmer temperatures. In general, seawater used for depuration procedure should be in the range between 14 and 29°C and should not vary by more than 20 °C from that of the seawater at harvest area (Neilson and others 1978).

Numbers of studies have been conducted to determine effect of water temperature on bacterial reduction by depuration (Hefferman and Cabelli 1970; Cabelli and Hefferman 1971; Souness and Fleet 1979; Rowse and Fleet 1984; Richards 1990; Rodrick and Schneider 1991a). Findings from these studies indicated that shellfish species from different geographical locations might pump at different ranges of temperatures. However, these ranges deviated only slightly from room temperature, which suggested that most shellfish could be depurated under room temperature. Table 2.3 lists optimal temperatures for reducing various types of microorganisms in shellfish by depuration reported by various studies.

Table 2.3 Optimal temperatures for reducing various types of microorganisms in shellfish by depuration (Data adopted from Hackney and Pierson 1994).

	Optimal Temperature (°C) of depuration
Hard Clam	
Fecal coliforms	22-25
E. coli	20
V. parahaemolyticus	15
V. cholerae	22-24
V. vulnificus	22-24
Eastern Oyster	
Fecal coliforms	24-29
Total coliforms	24-29
V. cholerae	22-24
V. vulnificus	22-24
Sydney Rock Oyster	
Total coliforms	18-20
E. coli	13-20
Salmonella spp.	18-22
Soft-shelled Clam	
Total coliforms	8-16
E. coli	8-16
Salmonella spp.	13

Salinity

Another critical factor for a successful depuration of shellfish is to maintain an optimal salinity in water. Changes in water salinity may affect the pumping rate and other physiological processes of shellfish. The pumping activity of shellfish may be turned off at very low salinities. Therefore, increasing salinity in water tends to improve the depuration process. Since the salinity of seawater used in the depuration process might be different from that of the harvest area, shellfish need time to be acclimated to the new environment before it begins active pumping again. In general, most shellfish species seem to depurate effectively at a salinity level of 30 ppt. It is recommended that the salinity of water used in depuration do not deviate more that 20% from that of the water at harvest area (U.S. Public Health Service 1987). Table 2.4 is a list of optimal salinities for reducing *E. coli* and coliforms in shellfish by depuration.

Table 2.4 Optimal salinities for reducing *E. coli* and coliforms in shellfish by depuration (Data adopted from Hackney and Pierson 1994)

Shellfish	Bacteria	Optimal Salinities (ppt) for
Hard Clam	*E. coli*	20-30
Eastern Oyster	Total coliforms	16-18
	Fecal coliforms	16-18
Sydney Rock Oyster	*E. coli*	23-26

Dissolved oxygen

Dissolved oxygen (DO) is required during depuration to maintain normal physiological activities of shellfish. The levels of DO in water can be influenced by several factors: (1) the surface area of depuration tank exposed to air; (2) flow rate of water during depuration; (3) shellfish loading density; (4) the physiological activity of shellfish during depuration; (5) aeration; (6) the temperature and salinity of the seawater used for depuration (Roderick and Schneider 1994). The DO level in the water should not fall below 50% saturation (Fleet 1978).

Turbidity

Turbidity is caused by presence of suspended particles in water (Gippel 1983), and is measured in nephelometric turbidity unit (NTU) (Kirk 1986). Excessive turbidity may affect the filtration rate of shellfish and reduce the ability of shellfish to effectively purge contaminants during depuration (Jackson and Ogburn 1999). The U.S. established a requirement for the turbidity of water for depuration to be less than 20 NTU in 1990 (NSSP, 1990). However, the requirement was modified by the NSSP in 1995 to "Turbidity does not exceed a value that will inhibit normal physiological activity of oysters and/or interfere with the process of water disinfection". Thus, no maximum turbidity level is set by the regulation, but a

maximum allowable level of turbidity must be established by each plant based on types and loads of suspended solids that might occur in water at different sites (Jackson and Ogburn 1999).

Methods of Water Disinfection

Seawater used in depuration need to be disinfected to prevent depurated shellfish from being contaminated with bacteria in the water. Several means including ultraviolet radiation and treatments of ozone, and chlorine can be used for disinfecting seawater (Otwell and others 1991).

Ultraviolet (UV) radiation

Ultraviolet radiation is widely used to disinfect water used in shellfish depuration in Australia, Denmark, Malaysia, New Zealand, Philippines, Singapore, United Kingdom, and United States. Many studies have reported that bacteria and viruses could be destroyed by UV light between 250 and 260 nm (Chang and others 1985; Fogh 1955; Harm 1980; Hill and others 1969, 1970). Exposure of microorganisms to UV light will result in damage of nucleic acids and lead to prevention of protein transcription and replication of microorganisms (Legan 1982). The major advantage of applying UV radiation for seawater disinfecting is that it

doesn't yield byproducts in the seawater. However, the bactericidal effects of UV light differ among bacterial species and the transmission of UV radiation into seawater is affected by turbidity and dissolved salts.

Ozone

Ozone is commercially used in France and Spain to disinfect seawater used for depuration. It is a strong chemical oxidant that can cause damage of nucleic acids and prevent the replication of bacteria (Roy and others 1981). It was reported that 1.1 mg/L of ozone could inactivate 10^4 oocysts of *Cryptosporidium parvum* within 6 minutes (Peeters and others 1989). However, ozone is also toxic to shellfish and should be removed from treated seawater before being used for depuration. Methods for preventing direct contact of shellfish with ozone in seawater during depuration include the control of ozone usage, resting treated water before use, converting residual ozone in seawater back to oxygen with UV radiation, and removing ozone with granular activated carbon (Bitton 1994). Application of ozone for disinfecting seawater offers some advantages over UV light radiation. Residual ozone at the level of 0.1-0.2 mg/L in depuration water could help the reduction of *Vibrios* and other pathogens in the gut of shellfish (Le Pauloue and others 1991).

Chlorine

Chlorine is the most widely used disinfectant and is commonly used as a disinfecting agent for depuration water in France, Italy and Spain. However, elevated levels of chlorine in depuration water could affect the pumping activity of shellfish and compromise the tastes of the shellfish (Blogoslawski 1991; Rodrick and Schneider 1991b). An early study reported that water containing approximate 0.2 ppm free chlorine could limit the filter-feeding activities of oysters (Dodgson 1928).

While depuration with clean seawater can be used to reduce bacterial contamination in oysters, it is ineffective in reducing *Vibrio* contamination in oysters due to the colonization of *Vibrio* cells in the digestive tracts. Studies have shown no significant reduction of naturally occurring *V. parahaemolyticus* in oysters after the depuration process (Eyles and Davey 1984), and it required 16 days to depurate laboratory-contaminated *V. vulnificus* in oysters to non-detectable level (Kelly and Dinuzzo 1985). However, replacing clean seawater with a solution exhibiting strong bactericidal effects might improve the efficacy of depuration process for reducing *Vibrio* spp. in contaminated oysters.

Electrolyzed Oxidizing (EO) Water

Electrolyzed oxidizing (EO) water, generated through electrolysis of a dilute salt solution, was recently introduced as a new anti-microbial agent. The EO water contains hypochlorous acid, low pH value, and high oxidation-reduction potential (ORP) and has been reported to exhibit strong antibacterial activities against many foodborne pathogens including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Bacillus cereus*, *Campylobacter jejuni*, and *Salmonella enteritidis* (Al-Haq and other 2001; Hayashibara and others 1994; Izumi 1999; Kim and others 2001; Park and others 2002a; Venkitanarayanan and other 1999a). Application of EO water as a disinfectant for reducing microbial contaminations has been reported for fresh fruits and vegetables (Izumi 1999; Koseki and others 2001; Koseki and others 2004a, b, c), poultry carcass (Park and others 2002a) and egg shell (Park and others 2005). Major advantages of using EO water as a disinfecting agent are easy to use and safe to workers. EO water can be produced on-site and used directly without dilution. Therefore, most hazards associated with handling concentrated chemicals can be avoided. An added benefit of using EO water instead of other sanitizing chemicals is that EO water is produced with water and NaCl without additional chemicals, thus has less adverse impact on the environment.

Production of EO Water

Production of EO water is conducted with diluted salt solution in a cell containing both positively and negatively charged electrodes separated by a membrane (Figure 2.1). When the electrodes are subjected to direct current voltage, two types of water with different characteristics are produced. An electrolyzed alkaline solution with high reduction potential is produced from the cathode side and called electrolyzed reducing (ER) water. The ER water could reduce the amount of free radicals in biological systems and has been reported to exhibit anti-oxidative effect on highly unsaturated fats and oils (Miyashita and others 1999). On the other hand, an electrolyzed acidic solution with high oxidation potential is produced from the anode side and called electrolyzed oxidizing (EO) water.

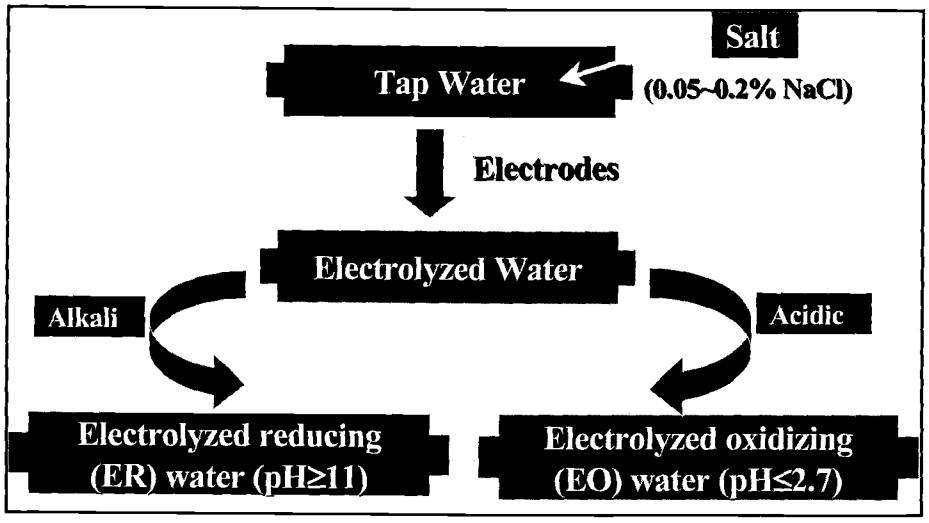

Figure 2.1 Schematic of EO water production

General properties of EO water include low pH, high oxidation-reduction potential (ORP), and chlorine in the water. The properties of EO water can be changed according to production parameters, such as salt concentration, voltage electrolyte flow rate, and temperature (Ezeike and Hung 2004). Table 2.5 lists properties of EO water produced using various setting of parameters. Studies conducted by Ezeike and Hung (2004) using response surface methodology (RSM) found that NaCl concentration was the most significant factor affecting pH, oxidation-reduction potential (ORP), and free chlorine content of the EO water produced. Besides the salt concentration, electrolyte flow rates and voltages can also be critical parameters to affect EO water properties. However, flow rate seemed to play a more important role than does voltage. In general, higher NaCl concentrations and voltages will allow the production of EO water with higher acidity and residual chlorine with greater ORP values. On the other hand, increasing electrolyte flow rate during EO water production will result in EO water with lower acidity and residual chlorine with smaller ORP values. This is caused by shorter electrolysis because of reduced residence time of salt water in the electrolytic cell.

Table 2.5 Production parameters affecting properties of EO water (data adopted from Ezeike and Hung 2004).

Salt (%)	Flow Rate (L/min)	Voltage (V)	Residual Chlorine (mg/L)	ORP (mV)	pH
0.0	0.5	2	0	404.9	5.84
		10	0	480.2	4.52
		28	0	613.6	4.27
	2.5	2	0	465.6	5.85
		10	0	467.4	5.30
		28	0	507.6	4.78
	4.5	2	0	440.6	5.58
		10	0	450.1	5.55
		28	0	458.2	5.15
0.1	0.5	2	0.25	752.0	3.97
		10	136.67	1144.7	2.32
		28	177.33	1143.9	2.18
	2.5	2	0	611.8	5.35
		10	34.50	1105.9	2.74
		28	56.67	1126.5	2.45
	4.5	2	0	559.1	4.82
		10	21.75	1085.1	2.99
		28	36.00	1116.2	2.56
0.5	0.5	2	2.50	960.3	3.95
		5	329.33	1134.6	2.32
		7	482.58	1140.8	2.07
	2.5	2	0	712.1	4.63
		5	88.67	1111.1	2.85
		7	121.42	1121.3	2.60
	4.5	2	0	566.4	4.95
		5	54.75	1096.0	3.04
		7	74.92	1107.6	2.83

Antimicrobial Mechanism

The properties of EO water contributing to the antimicrobial effect on pathogenic bacteria include pH, free chlorine, and oxidation-reduction potential (ORP). Hypochlorous acid (HOCl) is produced in EO water through electrolysis of salt water and plays an important role in EO water's antibacterial activities. Study conducted by Len and others (2000) found a strong correlation between concentrations of hypochlorous acid (HOCl) and the antibacterial activity of EO water, suggesting that HOCl was the primary antibacterial agent in EO water. It is believed that HOCl at its neutral charge can penetrate bacterial cell walls and interact with essential metabolic systems (White 1999). Increasing salt concentrations in water for electrolysis usually results in increased amounts of HOCl in the produced EO water and, therefore, allowing exhibition of stronger antibacterial activities (Anonymous 1997). During the salt water electrolysis, both HOCl and chloride ion (Cl^-) can be produced. Interaction between HOCl and Cl^- results in formation of chlorine gas.

$$NaCl + H_2O \longrightarrow Na^+ + Cl^- + H^+ + OH^-$$

$$HOCl + Cl^- + H^+ \longrightarrow H_2O + Cl_2$$

Loss of chlorine gas could result in reduced concentration of HOCl in EO water and lead to loss of antimicrobial activity. Therefore, EO water should be used right after

production or be kept in a closed container until use to avoid loss of antibacterial activity.

In addition to hypochlorous acid, high ORP value was also reported to contribute to the antibacterial activities of EO water. Aerobic bacteria generally require positive ORP in the range of +200 to +800 mV to grow, while anaerobic bacteria prefer negative ORP between -40 and -400 mV. While some studies reported that the available chlorine in EO water was the main agent for EO water's antimicrobial property (Oomori and others 2000), Kim and others (2000) suggested that the ORP was the primary factor for EO water's antibacterial activity.

The low pH of EO water has been reported to be a minor factor contributing to EO water's antimicrobial property. Most bacteria, except a few such as lactic acid bacteria, can only grow in limited pH range between 4 and 9 (Table 2.6). The pH of EO water is usually below 3.0 and, therefore, has negative effect on growth of many bacteria. A study conducted by Liu and other (2005) found that a treatment of EO water for 5 min reduced *L. monocytogenes* on clean stainless steel surface by more than 5-log CFU/chip, while a treatment of acidic water with same pH of EO water but much lower ORP and no chlorine could only reduce the bacteria by about 1.4 log CFU/chip. The study indicated that both the available chlorine and high ORP value are important to EO water's strong antibacterial activity.

Table 2.6 Minimum pH values for the growth of some foodborne bacteria (Data adopted form Jay 2005)

Aeromonas hydrophila	ca. 6.0
Bacillus cereus	4.9
Clostridium botulinum (proteolytic)	4.6
C. botulinum (nonproteolytic)	5.0
C. perfringens	5.0
Escherichia coli O157:H7	4.5
Lactobacillus brevis	3.16
L. plantarum	3.34
L. sakei	3.0
Lactococcus lactis	4.3
Listeria monocytogenes	4.1
Propioniibacterium cyclohexanicum	3.2
Plesiomonas shigelloides	4.5
Pseudomonas fragi	ca 5.0
Salmonella spp.	4.05
Shewanella putrefaciens	ca 5.4
Shigella flexneri	5.5-4.75
S. sonnei	5.0-4.5
Staphylococcus aureus	4.0
Vibrio parahaemolyticus	4.8
Yersinia enterocolitica	4.18

Application

Lettuce

Fresh fruit and vegetables may contain numerous microorganisms and some of them could lead to diseases in human (Beuchat 1996). Washing surfaces with clean water is the most common method used to reduce bacterial contamination on raw fruits and vegetables. Unfortunately, washing raw products with water may have limited effect on reducing total bacterial populations on the surfaces (Beuchat 1992; Brackett 1992; Nguyen-the and Carlin 1994). However, studies have shown that EO water could be used to wash fresh produce and reduce total and pathogenic bacteria. Washing lettuce in alkaline electrolyzed water for 1 min followed by in EO water for 1 min reduced viable aerobes by 2 logs CFU/g without damaging surface structure of lettuce (Koseki and others 2001). Washing lettuce leaf with EO water for 3 min significantly reduced population of inoculated *E. coli* O517:H7 and *L. monocytogenes* by 2.41 and 2.65 log CFU/g, respectively ($p<0.05$), without creating significant changes in quality of lettuce during 2 weeks' storage (Park and others 2001). The bactericidal effect of EO water against microorganisms on lettuce was also observed when ice made of EO water was use in study. Reduction of aerobic bacteria (1.5 log CFU/g) was observed when lettuce was kept in EO water ice and maintained at 2 °C to 3 °C for 24 h (Koseki and others 2002, 2004a).

Recently, studies have been conducted to determine combined effects of using both ER and EO waters. Although a practical use of ER water as a sole washing or sanitizing agent has not yet been developed, studies have found that ER water could be used as a pre-wash agent and is applicable for a combined usage with EO water (Koseki and others 2004c). Treating lettuce inoculated with *E. coli* O157:H7 and *Salmonella* with mildly heated (50°C) ER water for 5 min followed by washing with chilled (4°C) EO water for 1 min could lead to 3-4 log CFU/g reductions of both *E. coli* O517:H7 and *Salmonella* on the lettuce. Extending the time of the mild heat pre-treatment resulted in greater bactericidal effect than that observed by extending washing time from subsequent wash with chilled EO water. No deterioration of the lettuce was observed after the combined electrolyzed water treatment.

In addition to fresh lettuce, use of EO water as a potential sanitizer for reducing bacterial contamination on cucumbers was also studied. EO water was found effective on reducing coliform bacteria and fungi related to cucumbers. Treatment of EO water for 10 min also resulted in reduction of aerobic mesophiles naturally present on the cucumbers by 1.4 log CFU per cucumber. However, EO water treatment could not completely remove microorganisms from surface of cucumber due to surface structure properties (Koseki and others 2004b).

Fruits

Brown rot caused by *Monilinia fructicola* is one of the most destructive diseases of stone fruits (DeVries-Paterson and others 1991). Treatment of non-wounded peach inoculated with *Monilinia fructicola* in EO water was reported to be capable of reducing incidence and severity of the disease (Al-Haq and other 2001). Peach treated with EO water and held at 2°C with 50% relative humidity (RH) for 8 days did not develop brown rot until they were transferred to a storage condition of 20 °C with 95% RH. Immersion of peach in EO water for 5 min yielded the maximal reduction of incidence and severity of the disease. No chlorine induced phytotoxicity was observed on the treated fruits during the study.

Study on EO water as a potential disinfecting agent for strawberries found that aerobic mesophiles were reduce by less than 1 log CFU per strawberry after the EO water treatment (Koseki and others 2004b). Slightly greater reductions (1.0 to 1.5 log CFU) of coliform bacteria and fungi per strawberry were also observed in the study. Similar to results observed in cucumber study, EO water treatment could not completely remove microorganisms from surface of strawberry due to the surface structure properties.

Eggs

The egg surface is easily contaminated with pathogens, such *Salmonella* and *L. monocytogenes*, mainly from hen's feces and contaminated environments (Golden and others 1988; Humphrey 1994). Treatment of contaminated eggs with EO water reduced *L. monocytogenes* contamination on surface by up to 3.70 log CFU per shell egg (Park and others 2005). A combined treatment of one minute ER water followed by one min of EO water (41mg/L chlorine) yielded 4.01 and 3.81 log CFU reduction of *Listeria* and *Salmonella* per shell egg, respectively. When electrostatic spraying of EO water was used to disinfect shell eggs inoculated with high level of bacteria (much higher than that would be present in industrial situations), all bacteria on egg shells were completed eliminated (Russell 2003).

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 (Hood and others 1988; Humphrey and others 1993; White and others 1997). Recent study conducted by Park and others (2002a) found that washing chicken carcass with EO water (50 ppm chlorine content) at 23°C for 10 minutes could result in 3-log reduction of *C. jejuni* on the carcass. No viable

cells of *C. jejuni* were recovered in EO water after the wash.

Surface sanitizer

Kitchen counters and cutting boards are potential sources of cross-contamination of foods (Zhao and other 1998). Studies found that soaking cutting boards inoculated with *E. coli* O157:H7 in EO water reduced bacterial population by greater than 5.0 log CFU/100 cm^2, while a much less reduction of 1.0 to 1.5 log CFU/100 cm^2 was observed after soaking the boards in deionized water (Venkitanarayanan and other 1999b). The study also found a substantial reduction (about 5 logs) of *L. monocytogenes* on the cutting boards after EO water treatment.

The effects of EO water on reducing bacterial contamination on surfaces of glass, stainless steel, glazed ceramic tile, unglazed ceramic tile, and vitreous china have also been studied. Immersion treatments of those materials inoculated with *Enterobacter aerogenes* and *Staphylococcus aureus* in EO water for 5 min without agitation reduced populations by 2.2 to 2.4 log CFU/ cm^2 for *E. aerogenes* and by 1.7 to 1.9 log CFU/ cm^2 for *S. aureus*, whereas treatment with water only resulted in 0.1 to 0.3 log CFU/ cm^2 reduction for both bacteria (Park and others 2002). A similar study conducted by Liu and others (2005) found that populations of *L. monocytogenes* on clean stainless steel surface could be reduced to non-detectable

level after soaking in the EO water for 5 minutes.

Other Application

Food ingredients as well as microorganisms can attach to processing or surfaces equipments during food processing and need be removed completely by thorough cleaning and sanitation to minimize cross-contamination. ER water was reported to be more effective than warm water on removing gelatin on equipment surface with an optimal temperature for cleaning at 50 °C while EO water could be used to remove calcium hydrogen phosphate deposited on stainless steel particles (Fukuzaki and other 2004).

While the antibacterial activity of EO water against many pathogens have been reported, no study has been conducted to determine the effectiveness of EO water treatment on reducing *V. parahaemolyticus* and *V. vulnificus* contamination in seafood. Therefore, we conducted this study to investigate the antibacterial activity of EO water against *V. parahaemolyticus* and *V. vulnificus* and the potential application of EO water as a post-harvest processing to reduce *Vibrio* contamination in oysters.

CHAPTER 3

Effects of Electrolyzed Oxidizing Water Treatments on Reducing *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Raw Oysters

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Submitted to Journal of Food Protection

Abstract

Contamination of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oysters is a food safety concern. This study investigated the potential application of electrolyzed oxidizing (EO) water as a post-harvest process to reduce *V. parahaemolyticus* and *V. vulnificus* contamination in oysters. Freshly harvested Pacific oysters were inoculated with 5-strain cocktail of *V. parahaemolyticus* or *V. vulnificus* and held in EO water (chlorine: 30 ppm, pH: 2.82, ORP: 1131 mV) containing 1% NaCl at room temperature. Populations of *Vibrio* in treated oysters were determined at 0, 2, 4, 6, and 8 h. Treatment of laboratory-inoculated oysters with EO water resulted in significant reduction (>1.0 log MPN/g) of both *V. parahaemolyticus* and *V. vulnificus* in oysters after 4 h. The acidic and chlorine-containing water created an unfavorable growth environment for the oysters. Extended exposure (>12 h) of oysters to EO water containing >30 ppm chlorine was detrimental to oysters. A short-term (<6 h) EO water (30 ppm chlorine and 1% NaCl) treatment could be used as a post-harvest treatment to reduce *Vibrio* contamination in oysters.

Key Words: *Vibrio parahaemolyticus, Vibrio vulnificus*, electrolyzed oxidizing water, oysters, depuration.

Introduction

The United States produces more than 27 million pounds of oysters each year and most of them are sold and consumed raw without further processing (Hardesty 2001). Oysters can be easily contaminated with spoilage and pathogenic bacteria such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* through contaminated seawater. Growth of naturally contaminated bacteria in oysters during storage and retail sale results in loss of quality, reduced shelf life, and potential gastroenteritis (Fleet 1978).

V. parahaemolyticus and *V. vulnificus* are human pathogens that occur naturally in the marine environment (Ayres 1978; Dalsgaard 1998; DePaola and others 1990; Liston 1990). These organisms are commonly found in shellfish and are the leading causes of foodborne infections associated with seafood consumption in the U.S. (Andrews 2004). Recent outbreaks of *V. parahaemolyticus* infections associated with raw oysters consumption involving more than 700 illnesses in several regions of the U.S. (CDC 1998; 1999) indicate a need of developing effective post-harvest processes for reducing these pathogens in oysters for safe consumption.

Several post-harvest treatments including low temperature pasteurization, rapid chilling, freezing, high pressure processing, irradiation, and heat shock have been reported to be capable of achieving certain degrees of reductions of these

pathogens (Ruple and Cook 1992; Hesselman and others 1999; Schwarz 2000; Andrews and others 2001; Cook and Raghebeer 2001; Calik and others 2002). However, most of these processes require either significant amounts of initial investment or major effort on personnel training and oysters are often killed during the process.

Depuration is a controlled purification process allowing shellfish to purge sand and grit from the gut into clean seawater. The process usually leads to a reduction of microbial contaminants in shellfish and therefore increases shelf life of refrigerated products. However, studies have shown that depuration with clean seawater was not very 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; Kelly and Dinuzzo 1985). Therefore, it limits the usage of conventional depuration as a means for eliminating *Vibrio* contamination in oysters. Replacing clean seawater with a solution exhibiting strong anti-microbial activities might improve the efficacy of the depuration process for reducing *Vibrio* contamination in oysters.

Recently, electrolyzed oxidizing (EO) water generated through electrolysis of a dilute salt solution was introduced as a new anti-microbial agent. Studies have shown that EO water exhibited strong bactericidal effects against many foodborne

pathogens, including *Salmonella enteritidis*, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Escherichia coli* O157:H7 (Venkitanarayanan and others 1999a; Kim and others 2000; Kim and others 2001; Park and others 2001; Park and others 2002). Application of EO water as a disinfectant for reducing microbial contaminations has been reported for fresh fruits and vegetables (Izumi 1999; Koseki and others 2001; Koseki and others 2004), poultry carcass (Fabrizio and others 2002; Park and others 2002), and cutting boards (Venkitanarayanan and others 1999b). These results suggested that EO water might be used as a substitute of seawater in oyster depuration to increase the reduction of *Vibrio* contamination. This study investigated the anti-microbial activity of EO water against *V. parahaemolyticus* and *V. vulnificus* and the potential application of EO water as a post-harvest processing to reduce *Vibrio* contamination in oysters.

Materials and Methods

Bacterial cultures preparation. *V. parahaemolyticus* (10290, 10292, 10293, BE 98-2029, and 027-1c1) and *V. vulnificus* (93A3097, 93A4153, 96A6135, ATCC 27562, and DI27-3C) were used in this study. Each culture was individually grown in tryptic soy broth (TSB, Difco Laboratories, Sparks, MD) supplemented with 1.5% NaCl (TSB-Salt) at 37°C for 4-6 h. The cultures were streaked to individual tryptic

soy agar (TSA, Difco Laboratories) supplemented with 1.5% NaCl (TSA-Salt) and incubated at 37°C for about 24 h. A single colony was then selected from the TSA-Salt plate and enriched individual in TSB-Salt at 37°C for 4 h. The enriched cultures were pooled into a sterile centrifuge tube and centrifuged at 3,000 × g (Sorvall RC-5B, Kendro Laboratory Products, Newtown, CT) at 5°C for 15 min. Pelleted cells were resuspended in 50 ml of salt solution (2%) to produce a culture cocktail of approximate 8.7×10^8 cfu/ml.

Electrolyzed Oxidizing (EO) water production. EO water containing chlorine contents ranging from 10 to 50 ppm was produced with an electrolyzed water generator (Model V-500, Electric Aquagenics Unlimited, Inc. Lindon, UT) according to manufacturer's instruction. All EO water was produced on the day of experiments and used immediately after production.

Bactericidal activity of EO water against *V. parahaemolyticus* and *V. vulnificus*. The bactericidal activity of EO water [(1) chlorine:10 ppm, pH:3.17, ORP:1104 mV; (2) chlorine:30 ppm, pH:2.82, ORP:1131 mV; and (3) chlorine:50 ppm, pH:2.70, ORP:1139 mV] against *V. parahaemolyticus* and *V. vulnificus* was first determined by mixing 1.0 ml of the bacterial culture (approximate 8.7×10^8 cfu/ml) with 9.0 ml of EO water in a sterile tube. Survival of *V. parahaemolyticus* and *V. vulnificus* in EO water was determined at 15, 30, and 60 s after mixing by the

pour-plate method using TSA plates with serial dilutions in Butterfield's phosphate buffer (pH 7.2-7.4). The plates were incubated at 37°C for 48 h, and the colonies formed on plates were counted.

Effects of salt on bactericidal effects of EO water against *V. parahaemolyticus* and *V. vulnificus*. Effects of salt contents on bactericidal effects of EO water against *V. parahaemolyticus* and *V. vulnificus* were determined by mixing 1.0 ml of *Vibrio* culture cocktail with 9.0 ml of EO water (10, 30, or 50 ppm chlorine) containing various amounts of NaCl (0.5-2.0%). Survival of *V. parahaemolyticus* and *V. vulnificus* in EO water was determined at 15, 30, 60, 90, and 120 s with the pour-plate method using TSA-Salt plates and incubation at 37°C for 48 h. The most effective EO water and salt combination on inactivating *V. parahaemolyticus* and *V. vulnificus* was selected for oyster depuration studies.

Oyster Preparation. Freshly harvested oysters were obtained from Oregon Oyster Farm (Yaquina Bay, Newport, OR) and delivered immediately in a cooler with ice gels to the laboratory for experiments. The oysters were briefly washed with tap water to remove mud on shell and placed in artificial seawater (ASW, salinity: 29.6 ppt) at room temperature for 3-4 h before being inoculated with *Vibrio* spp. The ASW was prepared by dissolving Instant Ocean Salts (Aquatic Eco-System, Inc., Apopka, FL) with deionized water according to manufacturer's instruction.

Inoculation of oysters with *Vibrio* spp. Two levels of *Vibrio* contamination in oysters were prepared for studies. Oysters were transferred from the ASW to a tank of fresh ASW containing *V. parahaemolyticus* or *V. vulnificus* at a level of approximate 10^6 cfu/ml. The inoculation was conducted at room temperature overnight (12-14 h) with water being circulated at a flow rate of 11L/h. Air was pumped into the solution to facilitate colonization of *Vibrio* in oysters. A lower level of inoculation was conducted with ASW containing *V. parahaemolyticus* or *V. vulnificus* at a level of approximate 10^4 cfu/ml. Oysters and ASW were analyzed for *V. parahaemolyticus* or *V. vulnificus* contamination before the inoculation.

EO water treatment. Inoculated oysters were placed in a tank of EO water (30 or 50 ppm chlorine) containing 1% NaCl and transferred to freshly generated EO water every 1 h. The ratio of EO water to oyster was maintained at 1.0 liter EO water for every 4 oysters. The chlorine contents in EO water were determined with a commercial chlorine detection kit (HACH Company, Loveland, CO). The pH and oxidation-reduction potential (ORP) of EO water were measured with a pH meter (Model 420A, Orion Research, Inc., Boston, MA) and an ORP meter (CheckmateII Systems with Redox Sensor, Corning, Inc., NY), respectively. Inoculated oysters held in ASW were used as controls.

Microbiological tests. Populations of *V. parahaemolyticus* and *V. vulnificus*

in inoculated oysters held in EO water were analyzed before the treatment and at 2, 4, 6, 8, and 24 h with a 3-tube MPN method described in the Food and Drug Administration's Bacteriological Analytical Manual (USFDA 1998) using thiosulfate-citrate-bile salts-sucrose agar (TCBS) for *V. parahaemolyticus* and modified cellobiose polymyxin colistin agar (mCPC) for *V. vulnificus* determination. At each testing time, three oysters were randomly removed from the EO water tank and shucked with a sterile shucking knife in a sterile stainless tray. Each shucked oyster meat was placed in a sterile Whirl-Pak filter bag (Nasco, Modesto, California) and mixed with 9 volumes of sterile alkaline peptone water (APW). The oyster samples were homogenized with a stomacher (Seward Stomacher 400, Brinkmann, Westbury, NY) at 230 rpm for 1 min to prepare a 1:10 sample suspension. Additional ten-fold dilutions of each oyster sample were prepared with sterile APW. All sample dilutions were individually inoculated into test tubes containing alkaline peptone salt broth (APS). Inoculated APS tubes were incubated at 35-37 °C for 16-18 h and one loopful (3 mm) of enriched APS from the top 1 cm of a turbid tube was streaked onto individual TCBS for *V. parahaemolyticus* and onto mCPC for *V. vulnificus* detection. All plates were 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 or colonies that are round (1-2 mm diameter), flat, and yellow on mCPC were considered positive for *V.*

parahaemolyticus and *V. vulnificus*, respectively. Total populations of *V. parahaemolyticus* or *V. vulnificus* in samples were determined by converting numbers of APS tubes that were positive for *V. parahaemolyticus* or *V. vulnificus* to MPN/g using a MPN table.

Statistical analysis. Results of microbiological tests were transformed into log values for statistical analyses. Bacterial populations at different treatment times were analyzed with Two-sample *t* test (S-Plus, Insightful Corp., Seattle, WA). Significant differences between treatments were established at a level of p=0.05.

Results and Discussions

Bactericidal activity of EO water against *V. parahaemolyticus* and *V. vulnificus* cultures. EO water exhibited strong antibacterial activity against pure cultures of *V. parahaemolyticus* and *V. vulnificus*. Populations of *V. parahaemolyticus* (7.74 log cfu/ml) and *V. vulnificus* (7.69 log cfu/ml) decreased very quickly in EO water. No cells of *V. parahaemolyticus* or *V. vulnificus* were culturable (>6.6-log reduction) in the EO water (chlorine content: 10, 30, and 50 ppm) after being added to the water for 15 s.

Effects of salt contents on bactericidal effects of EO water against *V. parahaemolyticus* and *V. vulnificus*. Addition of salt to EO water containing low

level of chlorine (10 ppm) enhanced the survival of both *Vibrio* species in the water (Figure 3.1 & 3.2). While no culturable cells of *V. parahaemolyticus* or *V. vulnificus* were detected after 15 s in EO water containing a minimal amount of salt (0.5% NaCl), cells of both species were detected in EO water containing higher salt contents (1.0 % or higher) after 15 s of the treatment. *V. parahaemolyticus* were detected in EO water containing 1.0% NaCl at 60 s and in EO water containing 1.5% NaCl or higher at 90 s of the treatment (Figure 3.1). However, no culturable *V. parahaemolyticus* was detected in EO water containing 1.5 or 2.0% NaCl after 120 s. Similar results were observed for *V. vulnificus*. Cells of *V. vulnificus* were detected in EO water containing 1.5 or 2.0% at 30 s (Figure 3.2). However, no *V. vulnificus* was detected in any of the EO water after 60 s. These results indicated that *V. parahaemolyticus* was more resistant to EO water than *V. vulnificus* and addition of salt to EO water could decrease bactericidal effects of EO water against both *V. parahaemolyticus* and *V. vulnificus*.

It was not clear if the reduced bactericidal effect of EO water was caused by an interaction between NaCl and the anti-microbial components of EO water. Studies conducted by Liu and others (2005) demonstrated that the bactericidal activity of EO water against bacterial cells was mainly related to its chlorine contents. However, there was no apparent change in chlorine contents of EO water after the addition of

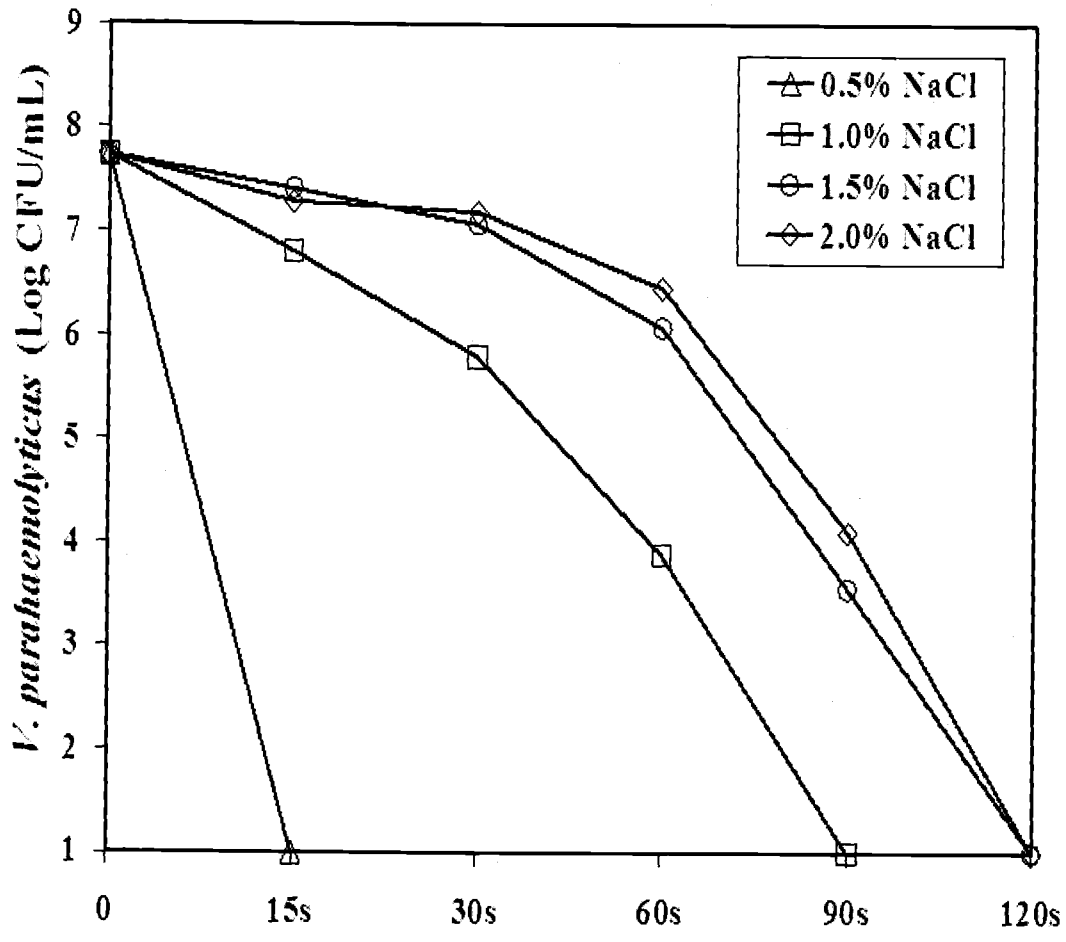


Figure 3.1 Survival of *V. parahaemolyticus* in EO water (chlorine: 10 ppm, pH: 3.17, ORP: 1104 mV) containing sodium chloride.

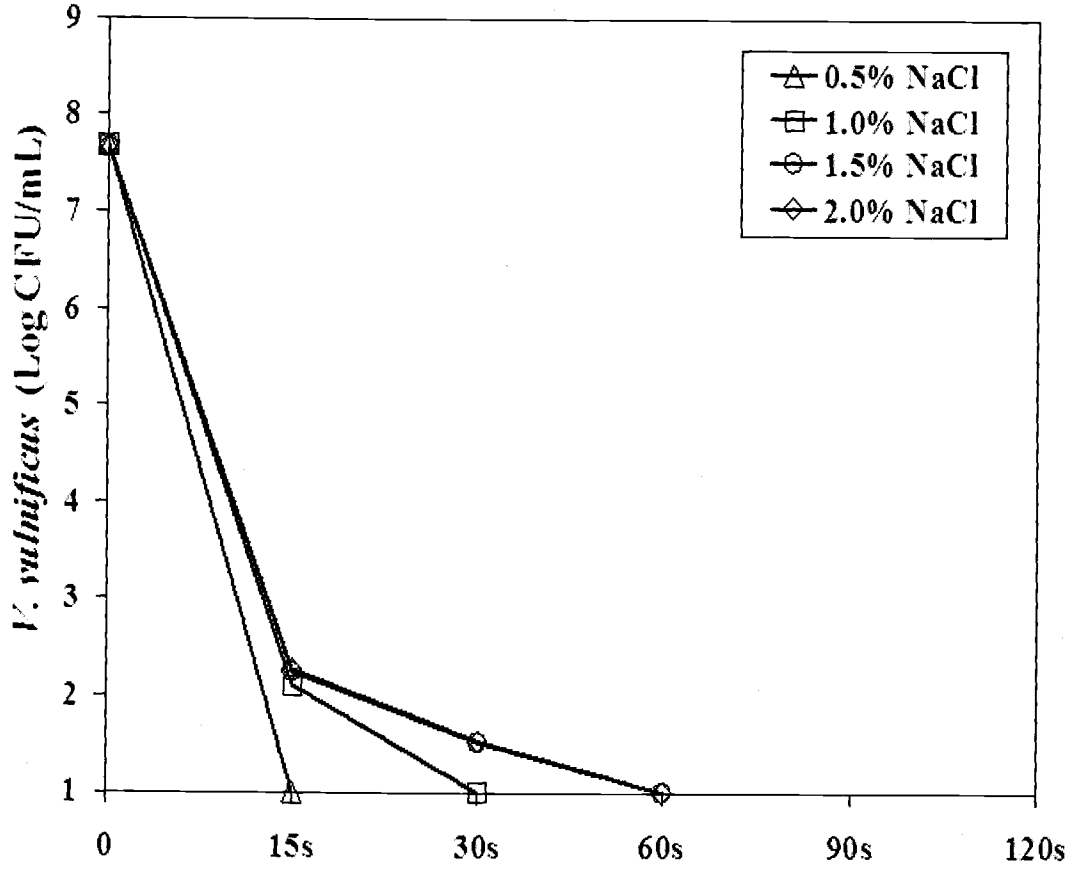


Figure 3.2 Survival of *V. vulnificus* in EO water (chlorine: 10 ppm, pH: 3.17, ORP: 1104 mV) containing sodium chloride.

salt to the water (data not shown). Another possibility is that the addition of salt to EO water enhanced the survival of both *V. parahaemolyticus* and *V. vulnificus* in the water. Both species were halophilic and require certain amounts of salt to grow.

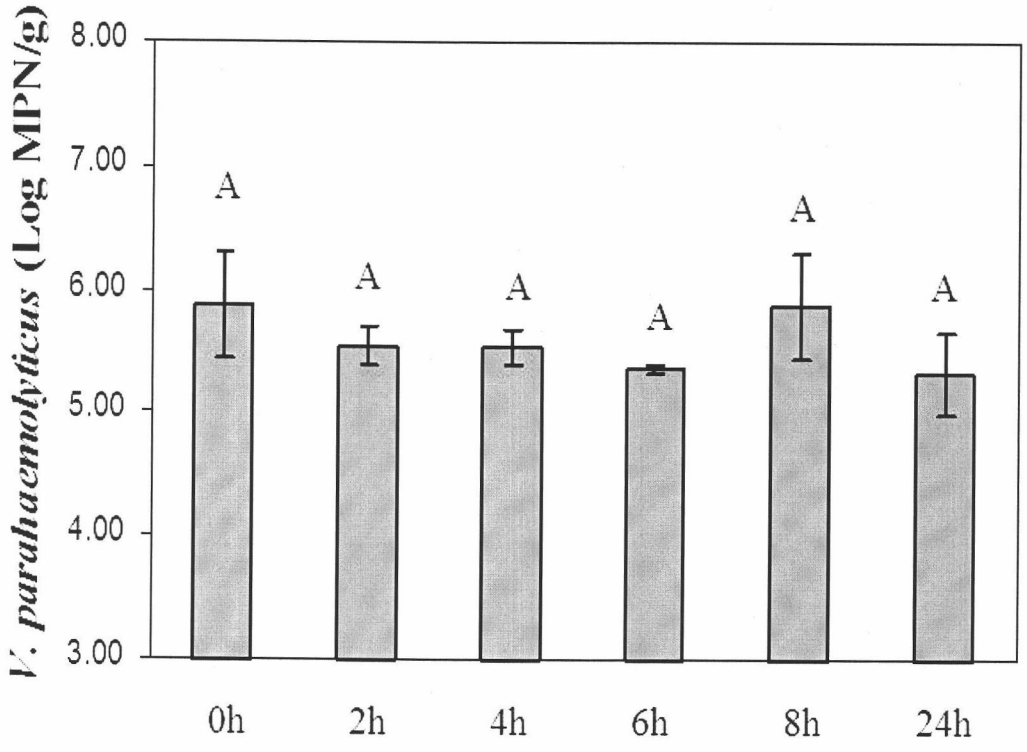
The reduced bactericidal effects of EO water against *Vibrio* cells due to addition of salt were not observed when the chlorine content in EO water increased to 30 or 50 ppm. No culturable cells of *V. parahaemolyticus* or *V. vulnificus* were detected in either water containing 2.0% NaCl after 15 s (data not shown). To eliminate the potential effects of salt on EO water's anti-microbial activity against *V. parahaemolyticus* and *V. vulnificus*, EO water contained higher chlorine (30 and 50 ppm) was used for processing oysters inoculated with *V. parahaemolyticus* or *V. vulnificus*.

Effects of EO water treatment on reducing *V. parahaemolyticus* and *V. vulnificus* in oysters. One concern of application of EO water for reducing *Vibrio* contamination in oysters is that oysters might not survive a long-term exposure to an acidic and chlorine-containing environment. Initial trials of holding oysters in EO water containing 50 ppm chlorine found that an extended exposure to the water was harmful to oysters. Many oysters died in the water after 12 h of exposure. However, oysters survived in EO water containing 30 ppm chlorine for more than 12 h. Therefore, EO water containing 30 ppm chlorine was selected for decontamination

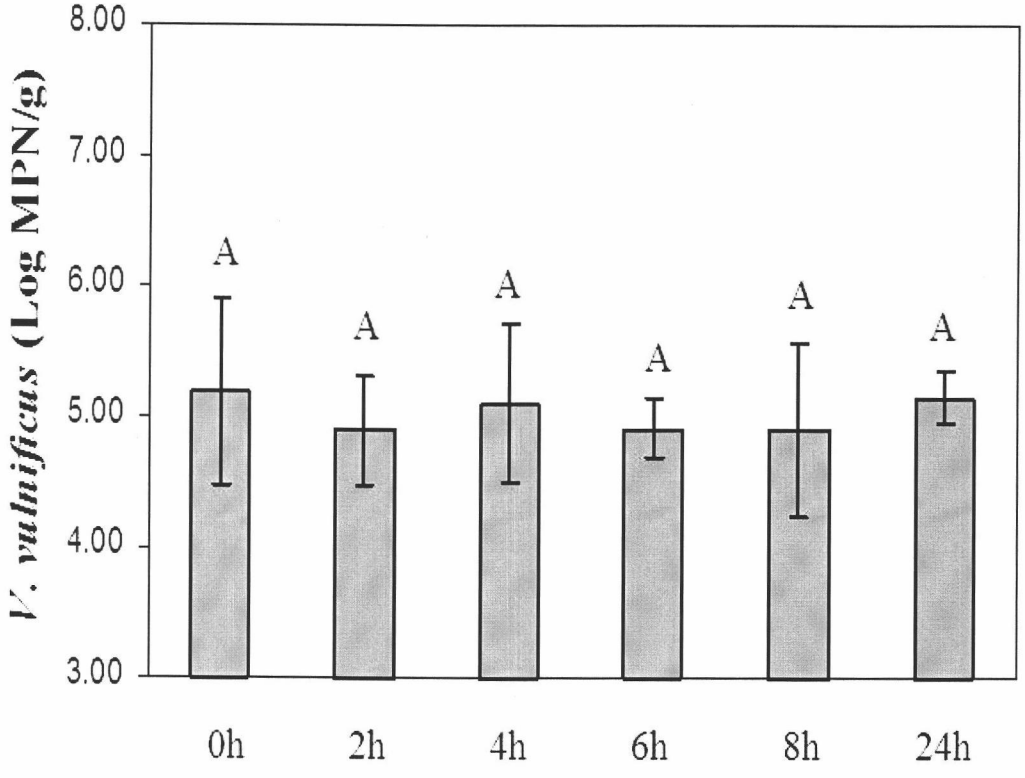
of oysters inoculated with *V. parahaemolyticus* and *V. vulnificus*.

Another big challenge of applying EO water to oyster processing is that the water needs to be circulated through the digestive tract of oysters so it can interact with bacterial cells and inactivate colonized pathogens. Since oysters are grown in the estuaries and marine environments, presence of salt in EO water might promote water filtration by oysters and allow the EO water to be circulated through the digestive tract. However, addition of salt to EO water was found to reduce the bactericidal effects of EO water against both *V. parahaemolyticus* and *V. vulnificus* (Figure 3.1 & 3.2). To minimize the negative salt effects, the concentration of salt in EO water (30 ppm chlorine) was set at 1% NaCl for the study.

Holding oysters in ASW for 24 h did not significantly reduce populations of *V. parahaemolyticus* or *V. vulnificus* in laboratory-contaminated oysters (Figure 3.3 & 3.4). This agrees with previous reports that depuration with clean seawater was ineffective in reducing *Vibrio* spp. contamination in shellfish. 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 level. However, holding contaminated oysters in EO water (30 ppm chlorine) for a few hours resulted in significant reductions of *V.*



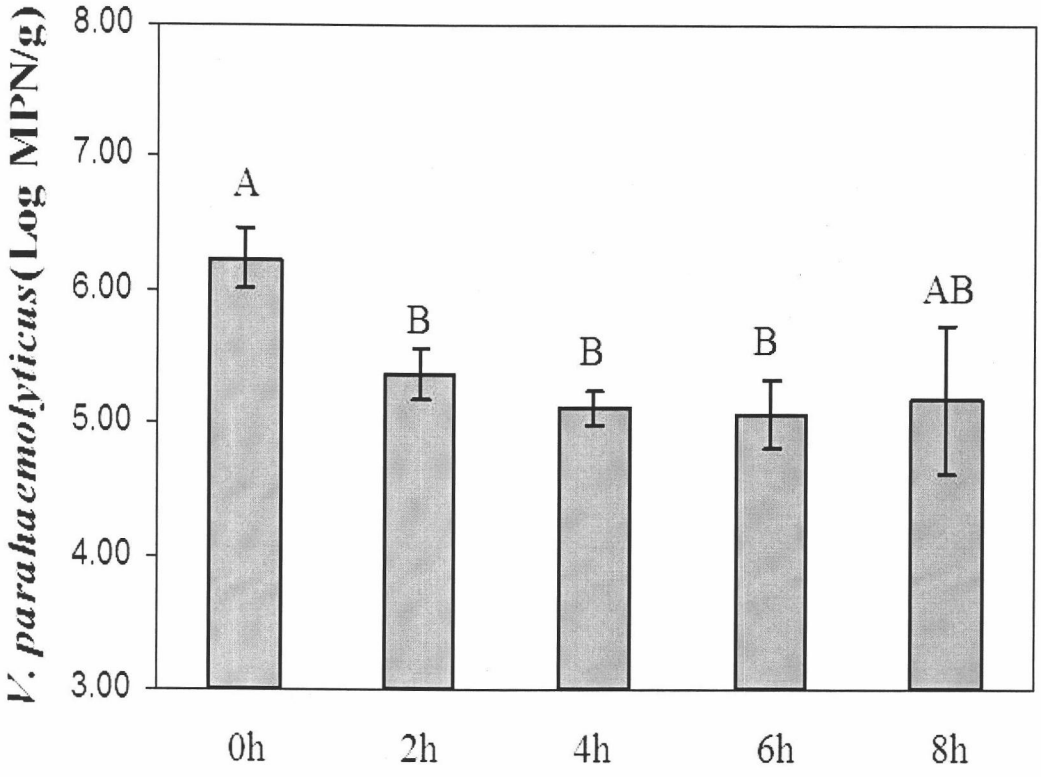
Figures 3.3 Effects of ASW depuration on reducing *V. parahaemolyticus* in laboratory inoculated oysters (initial population: Log 5.88 MPN/g). Data are means of 3 determinations ± SD. Means with the same letter are not significantly different ($p>0.05$).



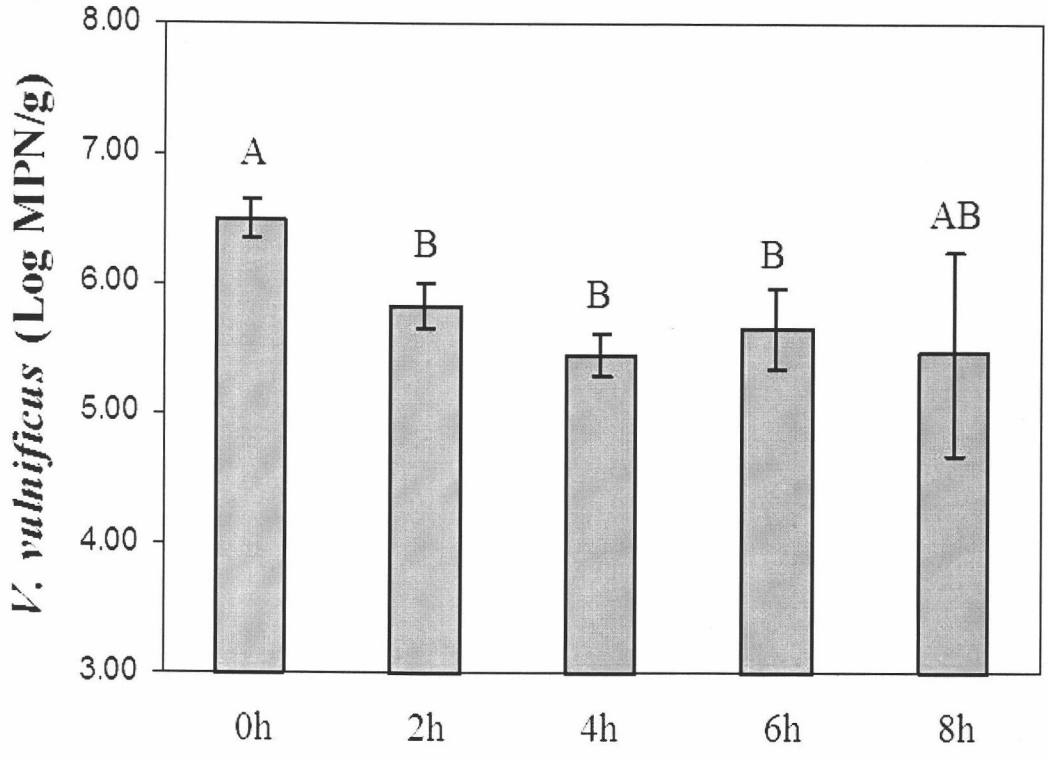
Figures 3.4 Effects of ASW depuration on reducing *V. vulnificus* in laboratory inoculated oysters (initial population: Log 5.19 MPN/g). Data are means of 3 determinations ± SD. Means with the same letter are not significantly different ($p>0.05$).

parahaemolyticus and *V. vulnificus* in oysters (Figure 3.5 & 3.6). Populations of *V. parahaemolyticus* were significantly reduced by 0.87 log cfu/g after 2 h of EO water treatment (Figure 3.5). The reduction increased to 1.13 log cfu/g after 4 h and remained at the same level until the end of 8 h. Similar results were observed when oysters inoculated with *V. vulnificus* were held in EO water containing 30 ppm chlorine and 1% NaCl. Populations of *V. vulnificus* in contaminated oysters were significantly reduced by 0.68 log cfu/g after 2 h. The reduction increased to the highest level (1.05 log cfu/g) after 4 h and remained at a similar level through 8 h (Figure 3.6).

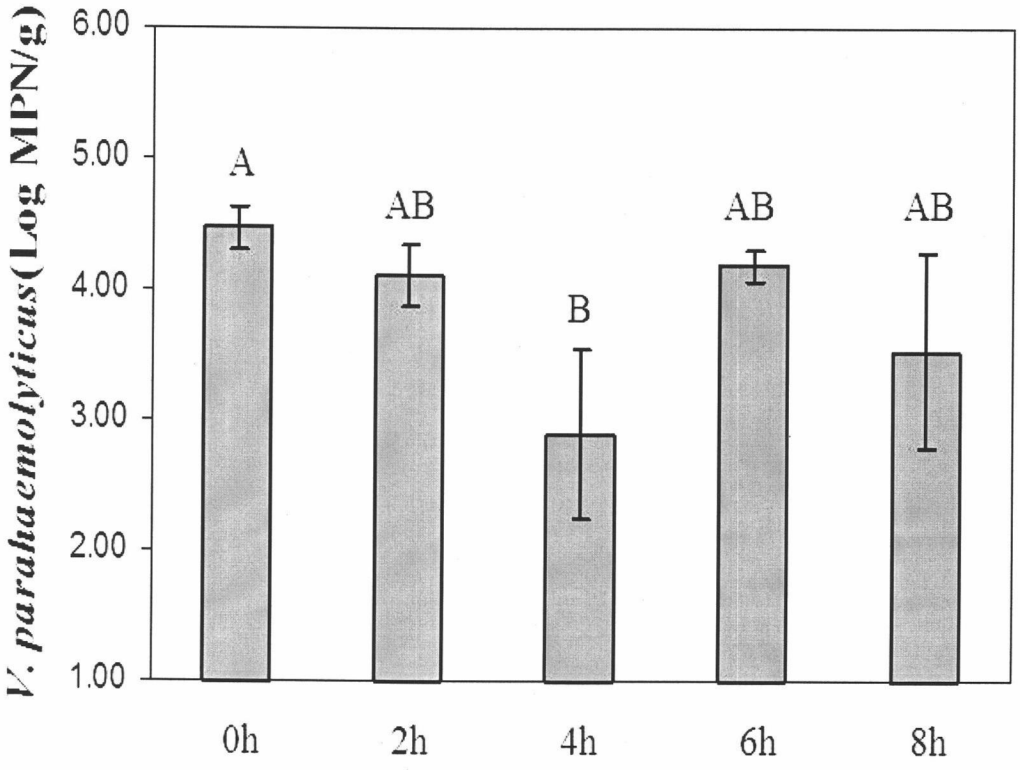
It is obvious that the acidity and chlorine of EO water create an unfavorable growth environment for oysters. It is believed that oyster was forced to stop its water-filtering activity after a few hours of exposure to EO water. This may explain the reduction of *Vibrio* cells in oysters reached the highest level after 4 h of treatment, but no further reduction was observed beyond that. Similar results were observed when oysters were inoculated with a lower level of *V. parahaemolyticus* (Log 4.47 MPN/g) and *V. vulnificus* (Log 4.0 MPN/g). Populations of *V. parahaemolyticus* and *V. vulnificus* in oysters were significantly reduced by 1.58 and 0.83 Log MPN/g after 4 and 8 h of EO water (30 ppm chlorine and 1% NaCl) treatments, respectively (Figure 3.7 & 3.8). It was not clear why the EO water treatment did not yield a



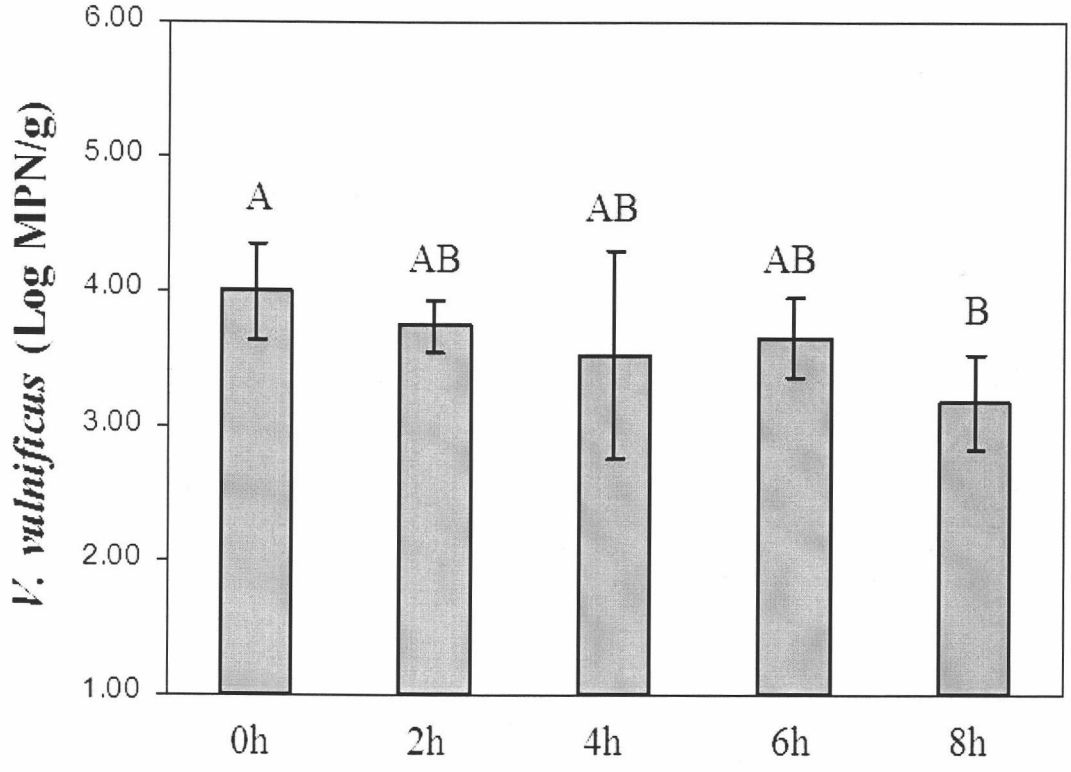
Figures 3.5 Effects of EO water treatment on reducing *V. parahaemolyticus* in laboratory inoculated oysters (initial population: Log 6.24 MPN/g). Data are means of 3 determinations $\pm$ SD. Means with the same letter are not significantly different ($p>0.05$).



Figures 3.6 Effects of EO water treatment on reducing *V. vulnificus* in laboratory inoculated oysters (initial population: Log 6.50 MPN/g). Data are means of 3 determinations ± SD. Means with the same letter are not significantly different ($p>0.05$).



Figures 3.7 Effects of EO water treatment on reducing *V. parahaemolyticus* in laboratory inoculated oysters (initial population: Log 4.47 MPN/g). Data are means of 3 determinations $\pm$ SD. Means with the same letter are not significantly different ($p>0.05$).



Figures 3.8 Effects of EO water treatment on reducing *V. vulnificus* in laboratory inoculated oysters (initial population: Log 4.00 MPN/g). Data are means of 3 determinations ± SD. Means with the same letter are not significantly different ($p>0.05$).

significant reduction of *V. vulnificus* in oysters until 8 h. A hypothesis is that this group of oysters was less active in filtering water but was able to continue the activity even at an unfavorable growth environment.

The exposure of oysters to EO water for an extended period was found to be detrimental to oysters. Although oysters were found to remain alive after being exposed to EO water containing 30 ppm chlorine for 8 h, many died after being held in the water for 24 h. It was not know whether the death of oysters upon a long-term exposure to EO water was caused by acidity or chlorine of EO water. The chlorine contents of EO water used in this study decreased from 30 ppm to about 15 ppm after 1 h, while pH value increased slightly by 0.2 - 0.3 units. There was no apparent change in oxidation-reduction potential (ORP) value during the treatment. Since chlorine is one of the major components contributing to EO water's anti-microbial activity, it is critical to keep the chlorine content in EO water at a level that is high enough to allow a reduction of *Vibrio* cells in oysters without killing oysters during the EO water treatment. Results of this study demonstrated that EO water containing 30 ppm chlorine could be used as a post-harvest process for reducing *V. parahaemolyticus* and *V. vulnificus* contamination in oysters. A same study conducted with EO water containing 10 ppm chlorine found no reduction of either *V. parahaemolyticus* or *V. vulnificus* after 8 h of treatment.

Conclusion

Contamination of *V. parahaemolyticus* and *V. vulnificus* in oysters could be reduced by EO water treatment. Holding laboratory-contaminated oysters in EO water containing 30 ppm chlorine for 4 h resulted in significantly reduction (>1.0 log MPN/g) of both *V. parahaemolyticus* and *V. vulnificus* in oysters. However, extended exposure of oysters to EO water was found to be detrimental to oysters. Treatment of oysters with EO water should be limited to 8 h or less. A short-term (4-6 h) EO water (30 ppm chlorine and 1% NaCl) treatment might be used as a post-harvest treatment to reduce *Vibrio* contamination in oysters.

CHAPTER 4

Conclusion

Depuration is a process used by the industry to reduce total bacterial populations in shellfish, particularly oysters. However, the process using clean seawater has been reported to be ineffective in reducing *V. parahaemolyticus* and *V. vulnificus* contamination in oysters because of special attachment of these pathogens in the digestive of tracts of oysters. This study investigated the antibacterial activity of electrolyzed oxidizing (EO) water against *V. parahaemolyticus* and *V. vulnificus* and the potential application of EO water as a post-harvest process to reduce contamination of *V. parahaemolyticus* and *V. vulnificus* in oysters.

EO water was found very effective on inactivating cells of both *V. parahaemolyticus* and *V. vulnificus* in pure cultures. Populations of *V. parahaemolyticus* (7.74 log cfu/ml) and *V. vulnificus* (7.69 log cfu/ml) decreased rapidly in EO water containing chlorine of 10 ppm or higher to non-detectable levels (>6.6-log reduction) within 15 s. However, holding raw oysters in EO water was found not as effective on inactivating cells of *V. parahaemolyticus* and *V. vulnificus* in raw oysters than in pure cultures.

The biggest challenge of applying EO water as a post-harvest process to reduce *V. parahaemolyticus* and *V. vulnificus* in oysters was that EO water need to be

filtered through the digestive tract by oysters so the *Vibrio* cells colonized in the digestive tracts could be inactivated by EO water. To promote the filtering activity of oysters, small amounts (1.0%) of salt (NaCl) were added to EO water for oyster treatments.

It is known that the chlorine content in EO water plays a major role in its antibacterial activity against bacteria. However, presence of chlorine in water used for depuration might create an unfavorable living environment for oysters. This study found that extended exposure of oysters in EO water containing high levels of chlorine (>30 ppm) was detrimental to oysters. Holding oysters in EO water containing 50 ppm chlorine for more than 12 h resulted in death of oysters. Reducing the chlorine concentration in EO water to 30 ppm reduced the toxic effect and allowed oyster to survive in the water for 24 h. While a further reduction of chlorine from 30 to 10 ppm in EO water would provide a friendlier environment for oyster depuration, our study of EO water containing 10 ppm chlorine found no reduction of either *V. parahaemolyticus* or *V. vulnificus* after 8 h in the water. Thus, EO water containing 30 ppm chlorine and 1% NaCl was selected for oyster treatments.

Holding oysters inoculated with *V. parahaemolyticus* or *V. vulnificus* in EO water (30 ppm chlorine and 1% NaCl) for a few hours resulted in significant reductions of both bacteria in oysters, while no apparent reductions of either bacteria

in inoculated oysters were observed after 24 h in artificial seawater. Populations of *V. parahaemolyticus* and *V. vulnificus* in oysters were significantly reduced by 0.87 and 0.68 log MPN/g, respectively, after 2 h of EO water treatment. The reduction increased to 1.13 log MPN/g (*V. parahaemolyticus*) and 1.05 log MPN/g (*V. vulnificus*) after 4 h and remained at similar levels until the end of 8 h.

It was obvious that holding oysters in EO water overtime had negative effect on oyster's filter-feeding activity. It is believed that oyster was forced to stop the activity after a few hours of exposure to EO water due to the low pH and presence of chlorine in the water. This explains that the reduction of *Vibrio* cells in oysters held in EO water reached the highest level after 4 h and no further reduction was observed beyond that. These results suggested that a short-term (4-6 h) EO water (30 ppm and 1% NaCl) treatment could be used as a post-harvest process for reducing *V. parahaemolyticus* and *V. vulnificus* contamination in oysters. However, more studies are needed to improve the efficacy of EO water treatment on reducing *Vibrio* contamination in oysters.

CHAPTER 5

Future Study

Future studies may be conducted to improve the efficacy of EO water treatments on reducing *V. parahaemolyticus* and *V. vulnificus* cells in contaminated oysters. One potential study could be the investigation of combined effects of artificial seawater (ASW) and EO water treatments on increasing efficacy of *Vibrio* reduction in oysters. Oysters will be treated with both ASW and EO water alternatively by switching oysters between EO water and ASW every few hours. The alternating treatments might help oysters to regain normal water-filtering activity after a few hours in ASW before being placed back in EO water. The treatment might also help oysters to survive better in an acidic and chlorine-containing environment and allow the removal of chlorine smell on oysters after EO water treatment. Finally, studies will need to be conducted to determine effects of EO water treatment on sensory characteristics of oysters.

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