AN ABSTRACT OF THE THESIS OF

Jenny K. Hansen for the degree of Master of Science in Food Science and Technology presented on May 20, 2002.
Title: Application of Ozone as a Disinfectant for Commercially Processed Seafood.

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Pacific oysters (*Crassostrea gigas*), Alaska pink salmon (*Oncorynchus gorbuscha*) roe and chum salmon (*Oncorynchus keta*) fillets were treated with aqueous ozone in both pilot plant and commercial settings to determine its effect on shelf-life and microbial changes. The microbial quality was analyzed by conducting pyschrotrophic and coliform plate counts on 3M petrifilm. Oxidative rancidity, pH and moisture were measured during the shelf-life study to determine the effects of ozone on quality. Concentrations of 0.5-1.3 ppm of ozone were applied for periods of 30 s, 1, 2 and 4 min at 5°C, 9°C and 15°C to determine an optimum ozone concentration and contact time that would decrease the seafood microbial load and increase shelf-life in the pilot plant studies. Oysters and roe were treated at 15°C and 5°C and fillets were treated at 9°C.

There where only minor microbial differences between ozonated and non-ozonated samples. Treatment temperatures rather than treatment types affected the microbial load. The pilot plant experiments at 15°C and 5°C showed 1 log decrease in oysters and roe treated with aqueous ozone at variable concentrations.

No increase in shelf-life was observed when salmon fillets were treated with aqueous ozone (1.3 ppm) for 2 min. Bacteria strains were isolated from treated (ozone and water) and control salmon fillet groups at 0, 5 and 10 days of storage and identified using the API20 NE system. The microbial change in the fillet flora did not differ between ozonated and non-ozonated treatment groups. Gram-positive bacteria were predominant in all groups at day 0. *Pseudomonas fluorescens* and *P. putida* were the predominant bacterial species found from all groups at days 5 and 10.

Aqueous ozone (0.5-1.7 ppm) was applied in a commercial Ikura roe processing facility. A decrease in the microbial load was seen in the pre-processed samples which were ozonated with eggs in the skein. There were no differences in microbial loads from the non-ozonated and ozonated post-processed samples of individual eggs removed from the skein.

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Application of Ozone as a Disinfectant for Commercially Processed Seafood

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APPROVED:

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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.

Jenny K. Hansen, Author

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DEDICATION

I would like to dedicate my thesis to my mother. Dr. Hansen-Johnson has been both my mother and teacher since the time I could walk. My mother would take me on some of her university field trips and there I listened to her marine biology lectures at age five. From the time I could remember my mother has continually taught both my brother and I about the world of science. I have a hard time thinking that I could have made it this far without the support, time and love of my mother.

APPLICATION OF OZONE AS A DISINFECTANT FOR COMMERCIALLY PROCESSED SEAFOOD

INTRODUCTION

Processing ready-to-eat and minimally processed foods using an effective, yet safe disinfectant is an important issue for the food industry. Minimally processed foods are processed with little or no heat with refrigeration or freezing being the sole means of preservation, both of which are not lethal to spoilage bacteria (Speck and Ray, 1997; Marth, 1998). Consumers are demanding an increase in quality, freshness and safety of foods (Foster and others, 1977; Holton, 2000; Khadre and others, 2001). One of the most highly perishable categories of food is seafood, with many products that are both minimally processed and manufactured as ready-to-eat. This category of seafood products often needs additional barriers against spoilage and contamination in addition to a decrease in storage temperature to $\leq 4°C$.

Washing seafood with chlorinated water is one of the most widely used disinfectant applications to retard bacterial contamination (Jantschke, 1992). In the last decade, chlorine has attracted negative attention due to the hazards associated with chlorinated by-products. Chlorination may lead to the formation of toxic or

carcinogenic organic compounds in water (Brungs, 1973; Tibbetts, 1995). Chlorine residues of >0.1 ppm could be excessive with respect to toxicity (Collins and Deaner, 1973). The potential hazard of carcinogenic trihalomethane compounds (THMs) in drinking water has lead to a regulated maximum contaminant level of 100 µg/L (Federal Register, 1997). Companies that produce minimally processed seafood products are searching for new methods that will decrease the microbial load and can be easily modified for installation into already existing processing lines. The use of aqueous ozone as an alternative to chlorine for seafood processing has been investigated as a possible alternative.

Studies with ozone and seafood have produced mixed results, but suggest considerable potential for seafood applications. Kötters and others (1997) treated rockfish (*Sebastes* spp.) with ozonated water in refrigerated seawater systems aboard a vessel. It was observed that ozonation enhanced detachment of surface bacteria and the shelf-life of the fish was extended 36 h. However, when the experiment was simulated in the laboratory, contrasting results were produced. The shelf-life of Atlantic cod (*Gadus morhua*) was not extended when stored on ozonated ice (Ravesi and others, 1987). However, Nelson (1982) demonstrated the benefits of ozonated ice by observing a microbial reduction of 97% from stored Alaskan salmon (*Oncorhyncus* spp.). Silva and others (1998) observed that fresh scad (*Trachurus trachurus*) treated with gaseous ozone, had one log reduction in total bacteria, from the fish skin samples. Ozone was ineffective as a bacteriocidal

treatment for shrimp (*Penaeus monodon*) that had been inoculated with nine different spoilage and pathogenic bacterial species (Chen and others, 1992).

The objective of this study was to determine the potential use of aqueous ozone in commercial seafood processing. Pacific oysters, salmon roe and salmon fillets were the seafood products used in this study. The specific goals were to determine whether there is an optimal ozone concentration and contact time that would decrease the seafood microbial load and increase shelf-life. The questions surrounding oysters, roe and fillets were investigated by attempting to create effective ozone systems that simulated industrial processing. To provide a realistic industrial application of each seafood product, pertinent seafood facilities were visited to observe functioning processing equipment. These observations aided in the experimental design of an aqueous ozone wash system that could be modified to fit an existing processing facility. They also gave insight into the realistic application of ozone use as a disinfectant in the seafood industry.

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CHAPTER 2: LITERATURE REVIEW

HISTORY OF OZONE

Ozone was discovered in the mid 1800s and used as a water disinfectant on a commercial basis in Europe before 1900 as shown in Table 2.1.

Table 2.1. Early history of ozone development (adapted from Brink and others, 1991).

Date	Significance
1840	Ozone was first discovered by Schönbein using electrolysis of sulfuric acid.
1891	Pilot plant tests in Germany showed ozone was effective against bacteria.
1893	The first full-scale water treatment by ozone disinfection occurred in the Netherlands
1897	A French chemist, Marius Paul Otto, wrote the first thesis on ozone and received his doctorate degree from La Sorbonne University in Paris.
1897	Otto also started the first company to manufacture and install ozone equipment for disinfection.
1898	Water disinfection facilities using of ozone began to be constructed throughout Europe.

The use of ozone for water purification was soon implemented on a global basis in several regions including North America, Africa, the Middle East and Asia. During the pre-World War I era, water was beginning to be treated with ozone for applications other than bacterial disinfection purposes. Full-scale application for removing iron and manganese from water occurred when engineers reported the oxidizing capabilities of ozone for water treatment (Vosmaer, 1916). During the 1960s, ozone was used primarily to removed iron and manganese from water in France and Germany (Brink and others, 1991). In the United States, the use of ozone was restricted and used mainly to remove metals as well as odors in water (O`Donovan, 1965). In 1982, the Food and Drug Administration (FDA) recognized that ozone was generally recognized as safe (GRAS), but with limitations (Federal Register, 1982). The FDA allowed the use of ozone as a disinfectant only in bottled water. A Food Additive Petition (FAP) was required for use of ozone in a food application (Federal Register, 2000). The Electric Power Research Institute (EPRI) filed an additive petition in response to FAP in September 2000 and the FDA approved the use of ozone for the treatment, storage and processing of foods (Federal Register, 2001).

BACTERIAL INACTIVATION MECHANISMS OF OZONE

There are several theories regarding the bacterial inactivation mechanism(s) of ozone. Most of the inactivation theories can be grouped into four general categories: cellular membrane components, enzymes, sulfhydryl bonds and genetic material. Research that supports ozone reactions at or in the cellular membrane components either suggest inactivation of key components at the cellular membrane or propose a series of inactivation areas. Giese and Christensen (1954) suggested that the surface of a bacterium was the primary inactivation site of ozone. Scott and Lesher (1963) proposed the site of ozone attack was at the double bonds of the unsaturated lipids in the cell envelope. Murray and others (1965) reported the lipoprotein and lipopolysaccharide layers of gram-negative bacteria as the primary area of attack by ozone, causing a change in cell permeability leading to cell lysis. Singer (1990) agreed with this hypothesis and mentioned specific sites, fatty acid double bonds, in the cell wall that could be oxidized. Komanapalli and Lau (1996) also reported that membrane components are the primary targets of ozone oxidation and suggested that subsequent reactions took place involving the intracellular components, protein and DNA. A study on *Pseudomonas fluorescens* and *Alcaligenes faecalis* showed that ozone initially targeted bacterial membrane glycolipids, glycoproteins or certain amino acids such as tryptophan and acted on the sulfhydryl groups of certain enzymes, resulting in disruption of normal cellular

activity (Greene and others, 1993). In a study on the inactivation of *Escherichia coli* treated with ozone, 95.5% of cells had altered membrane permeability (Santorum and others, 1999).

Two studies, on the inactivation mechanism(s) of ozone on *E. coli*, showed the primary inactivation mechanism was enzymatic in nature. Ingram and Haines (1949) found a general destruction of the dehydrogenation enzyme systems and proposed that cellular death may have resulted from interference with the respiratory system. Takamoto and others (1992) suggested that ozone caused a rapid decrease in β-galactosidase activity in the cytoplasm as well as decreased alkaline phosphatase activity in the periplasm of *E. coli.* Another possible inactivation site is the sulfhydryl bonds in the bacterial cells. These covalent bonds are formed between cysteine residues and give the cell secondary structural support. Sulfhydryl bonds are found in the polypeptide chains within the structural proteins in the cell wall. Barron (1954) suggested that oxidation of sulfhydryl groups (SH- to S-S) in the enzyme is the principle cause of cellular death. Other studies suggested that the sulfhydryl group in the membranes is the primary target of ozone attack (Mudd and others, 1969; Komanapalli and others 1997).

Hamelin and others (1977) reported the destruction of *E. coli* DNA polymerase I and the formation of DNA-protein crosslinks. After exposure to ozone, open circular DNA developed from closed circular plasmid DNA (Ishizaki and others, 1987). l'Herault and Chung (1984) reported that ozone may cause

mutations in *E. coli*, however, the same results were not found when *Salmonella* spp. were exposed to ozone (Victorin and Stahlberg, 1988).

Chen and others (1992) suggested that all cellular effects of ozone are concentration dependent. Bablon and others (1991) suggested that the large number of reactive sites on a cell causes different kinds of cellular inactivations. Several factors could affect variation among inactivation mechanisms and these include: strain of microorganism, age of the culture, number of microorganisms treated, presence of ozone-demanding media components, method of ozone application and method and accuracy of ozone measurement (Khadre and others, 2001).

OZONE GENERATION

There are many methods of ozone generation and the most extensively used is the corona discharge method (Kim and Yousef, 1999). Numerous studies in water purification and food processing systems utilized the corona discharge method for ozone generation technology (Colberg and Lingg, 1978; Sheldon and Brown, 1986; Takamoto and others, 1992; Kötters and others, 1997; Pryor and Rice, 1999; Richardson and others, 1999; Achen and Yousef, 2001). A corona discharge is achieved by forcing a high voltage of alternating current across a

discharge gap in the presence of air or oxygen (Fig. 2.1). A corona develops in the interstitial space of the discharge gap.

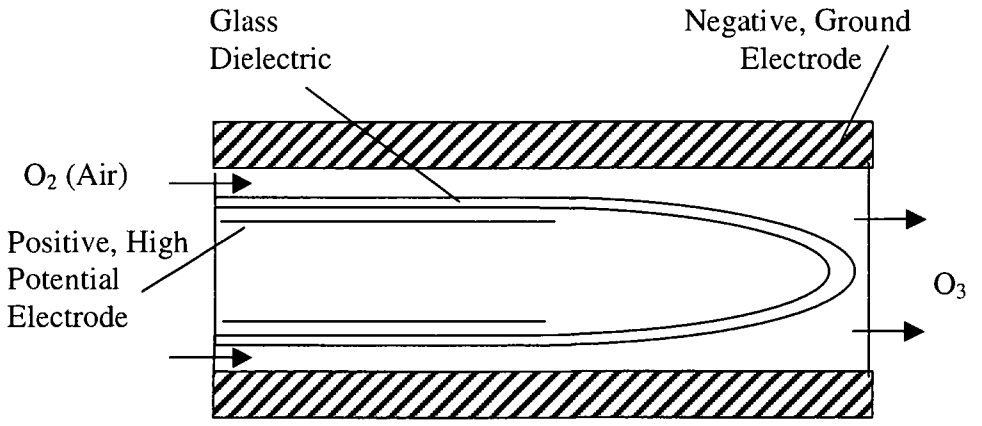

Figure 2.1. Corona discharge gap.
The electrodes are separated by a dielectric insulator and air gap. The electric current will not arc between the electrodes because of the dielectric and the air gap. When oxygen is excited by an electrical charge a splitting of the oxygen molecule occurs. The atoms split from the excited oxygen recombine with other (non-excited) oxygen molecules to form ozone (Ewell, 1950; Bablon and others, 1991; Pryor and Rice, 1999).

The high voltage excites the air and atoms split from the O_2 and combine with other O_2 molecules to form O_3 or ozone (Pryor and Rice, 1999) as described below:

$$3O_2 + \text{Energy} \rightarrow 2O_3 + \text{Heat} + \text{Light}$$

A potential disadvantage of the corona discharge method is the formation of nitric acid (Rosen, 1972). When moist air enters the corona discharge process, nitric acid is produced and results in corrosion. The amount of nitric acid formation can be decreased by the use of dry air in the system. The formation of nitric acid (Bablon and others, 1991) is:

$$O_2 + N_2 \rightarrow 2NO$$
$$2NO + O_3 \rightarrow N_2O_5$$
$$N_2O_5 \rightarrow 2NO_2 + \tfrac{1}{2}O_2$$
or
$$N_2O_5 \rightarrow NO + NO_2 + O_2$$
and
$$N_2O_5 + H_2O \rightarrow 2HNO_3$$

Other methods either produce ozone at a concentration that is too low for most commercial uses or the technology is not cost effective. The use of radiation to produce ozone has been used though the margins of applications are small due to its low ozone concentration. Low concentration of ozone (0.03 ppm) was produced from oxygen in the air by radiation at 185 nm emitted by high transmission UV lamps (Ewell, 1946). The electrochemical method splits water into hydrogen and oxygen atoms by electrolysis and the oxygen combines to form ozone and diatomic oxygen (Lynntech, Inc., 1998). Achen and Yousef (2001) used the electrochemical process to produce ozone in a study on apples inoculated with *E. coli* O157:H7.

Other ozone generation methods include: chemical, thermal, chemo-nuclear and electrolytic methods (Horvath and others, 1985).

METHODS OF OZONE MEASUREMENT

Eight methods for determining residual ozone in water are listed in Table 2.2. Grunwell and others (1983) recommended the indigo and arsenic (III) direct oxidation methods to measure aqueous ozone. The indigo method (Bader and Hoigne, 1982) is subject to fewer inconsistencies than other types of colorimeteric methods (Kim and Yousef, 1999). With the stoichiometery of 1:1 at a pH of 2, ozone reacts with the indigo trisulfonate dye at the double carbon bond (Fig. 2.2), which causes the dye to decolorize. The bleaching of the sulfonated dye is directly proportional to the concentration of ozone, which can be measured using a spectrophotometer at an absorbance of 600 nm. The absorbance decreases in a linear fashion with an increase in ozone concentration (Bader and Hoigne, 1982). Several studies have utilized the indigo method to measure ozone in aqueous solutions (Sheldon and Brown, 1986; Korich and others, 1990; Liltved and others, 1995; Kim and Yousef, 2000; Achen and Yousef, 2001).

Table 2.2. Ozone detection methods (adapted from Grunwell and others, 1983).

Iodometeric Methods			
	O_3 Reduction		I_2 Titration
Method	Excess Reagent	pH	pH
Iodometric	none	3.5-9.0	2
Amperometric	S_2O_3=	4.5	4.2-6.8
	PAO or As (III)		
As (III) Back Titration	As (III)	6.8	6.8
DPD	DPD	6.4	6.4
Non-Iodometric Methods			
Method	Reductant	pH	
Indigo	C=C	2	
As (III) Direct Oxidation	As (III), PAO	6.5-7.0	
Delta Electrode	e^-		
UV	none		

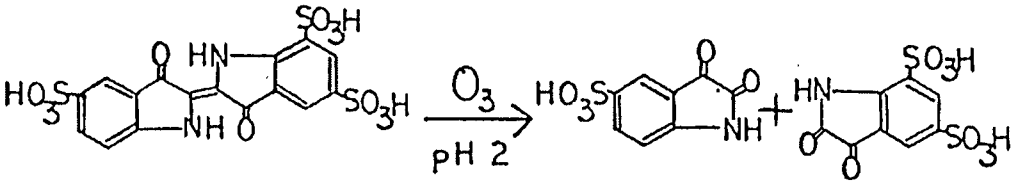

Figure 2.2. Decolorization of trisulfonate (Grunwell and others, 1983).

OZONE SOLUBILITY

Ozone is an unstable allotropic form of gaseous oxygen composed of 3 oxygen atoms (molecular weight of 48), a gas density of 2.144 g/L at 0°C and a boiling point of -122°C at 1 atm (Merkulova and others, 1971; Harrison, 1999). The stability of ozone varies with state, pH and temperature of the molecule due to molecular instability of the third oxygen atom. The half-life of gaseous ozone is between 4-12 h whereas the half-life of aqueous ozone can vary from seconds to hours depending on temperature and pH. In general, the half-life of ozone in distilled water at 20°C is 20-30 min (Khadre and others, 2001). Ozone is most stable in high purity water at pH <6; increasing pH above 7 induces hydroxyl free radical formation as ozone decomposes (Kim, 1998).

The solubility of ozone decreases as the amount of the organic load increases in a solution. Ozone solubility peaked at 3.8 ppm in distilled water and 1.4 ppm in the solution of shrimp meat extract (SME) after flushing the solutions with ozone for 27-35 min at 100 mL/min. The decomposition rate of ozone at 5°C in distilled water is 0.31/min and increased 8.6 times to 2.66/min in a shrimp-meat extract solution (Chen and others, 1992). Ozone has many decomposition pathways in tap water, each with its own types of kinetics (Staehelin and Hoigen, 1985). Decompostion rate of ozone can be calculated from the equation (Chen and others, 1992):

$$\text{Decomposition Rate} = \frac{\text{(Ozone conc. decreased x 100)}}{\text{Ozone conc. right after flushing stopped}} / \text{Time of Decomposition}$$

Ozone does not substantially react with pure water therefore it forms a true physical solution (Horvath and others, 1985). The solubility of ozone changes with differing chemical solution compositions. Ozone is more soluble in water than oxygen with solubility ratios of 0.64 and 0.049 at 0°C under 1 atm. Ozone solubility decreases with increasing temperature. The solubility of ozone in distilled water and shrimp meat extract (SME) at 5°C was 3.8 ppm and 1.4 ppm and decreased to 2.3 ppm and 0.6 ppm at 25°C (Chen and others, 1992). Therefore, a higher ozone concentration can be achieved in a solution with a low organic load at 5°C than in a solution with a large organic load at a temperature of 25°C when the solutions are being flushed with the same flow rate of ozone. The solubility ratio for ozone increases with decreasing temperatures as the apparent Henry's constant decreases with decreasing temperatures where Ha is the apparent Henry's constant ($atm/0_3$ molar fraction in liquid) and T is temperature (Ouederni and others, 1987):

$$\ln Ha = 22.3 - 4030/T$$

Ozone solubility is also influenced by pH (Gabovich, 1966; Hewes and Davison, 1973; Yang and Chen, 1979). As pH increases, the apparent Henry's constant increases and the solubility ratio decreases.

MECHANISMS OF OZONE DECOMPOSITION

Aqueous ozone decomposition is complex and depends on numerous factors including: the types of radicals formed (Grimes and others, 1983), types of organic matter that initiate, promote or inhibit the radical chain (Staehelin and Hoigne, 1982), pH (Jans and Hoigne, 1998), temperature (Nebel, 1981), and solution composition (Hoigne and Bader, 1975).

Aqueous ozone decomposes in steps forming a series of intermediates (Stumm, 1958) starting with hydroperoxyl ($^{\cdot}HO_2$), which degrades to hydroxyl ($^{\cdot}OH$) and finally to superoxide ($^{\cdot}O_2^-$) radicals. The types of compounds that are found in aqueous ozone solutions influence the pathway of ozone decomposition. If an ozone demand-free solution was seeded with microorganisms, the only demand on the ozone would be the microorganisms. Ozone will react differently when in contact with microorganisms that have been introduced to an unfiltered ozonated solution. The ozone may interact directly (oxidation) with the microorganisms or it

may start to break down and form other free radicals (ionization), each decomposition pathway with its own type of kinetics (Staehelin and Hoigne, 1982). In the free radical chain decomposition of ozone, initiator compounds (hydroxyl ions) can induce the formation of the superoxide ion from an ozone molecule. Promotor compounds regenerate the superoxide ion from the hydroxyl radical and inhibitor compounds (tert-butanol) consume ·OH without restoring the superoxide ion (Bablon and others, 1991).

Temperature and ozone solubility have an inverse relationship; an increase in temperature corresponds to a decrease in ozone solubility (Perrich and others, 1975; Bablon and others, 1991; Hunt and Marinas, 1997). Yang and Chen (1979) observed ozone in water at 2°C was more stable than at 25°C. Ozone decomposition increases with increasing pH (Harrison, 1999). At high pH levels, aqueous ozone decomposes rapidly due to the catalytic activity of the hydroxyl ion (Adler and Hill, 1950). The hydroxyl ion is an initiator of the free radical chain decomposition of ozone and will initiate free radical chain formation at high pH levels (Jantschke, 1992). At pH >7, hydroxyl free radicals are formed:

$$O_3 + H_2O \rightarrow O_2 + {\cdot}OH + HO^-$$

Hydroxyl radicals (2.76 V) are a stronger oxidizing agent than molecular ozone (2.076V) (Harrison, 1999). Even though hydroxyl radicals are stronger,

ozone is a far more stable compound and has a half-life extending between seconds and hours (depending on pH and temperature), while hydroxyl radical decomposition happens within microseconds. The hydroxyl free radical is an important chain-promoting radical. When the radical is not in a demand-free solution, it is often consumed preferentially by dissolved species found in the solution before it is encountered by dispersed particles such as microorganisms (Kim and Yousef, 1999).

Two general oxidation reaction pathways of aqueous ozone on organic compounds in water are 1) oxidation by molecular ozone where M is an organic compound:

$$O_3 + M \longrightarrow M_{ox}$$

and 2) ionization by free radical species formed from the decomposition of ozone (Staehelin and Hoigne, 1985; Jantschke, 1992; Hunt and Marinas, 1997):

$$O_3 \xrightarrow{OH^-} OH \xrightarrow{M} M_{ox}$$

Organic compounds (M) through either pathway will combine with ozone (M_{ox}). The factors leading to each reaction pathway are not well understood. It is difficult to predict how ozone reacts in the presence of different types of organic matter. Hunt and Marinas (1997) reported that in both the presence and absence of the

radical scavenger tert-butanol, molecular ozone was responsible for the inactivation of *E. coli*. These results are in agreement with other studies that used tert-butanol (Finch and others, 1992; Labatiuk and others, 1994). Others have suggested the opposite, that indirect ionization reactions by free radical species were responsible for inactivation (Dahi, 1976; Bancroft and others, 1984).

APPLICATION OF OZONE

Comparing ozone application in food systems is difficult due to the differing experimental designs of each study (Kim and Yousef, 1999; Khadre and others, 2001). The amount of microbial inactivation depends on the demand of the ozone (Kim and Yousef, 2000) and the type of solution that is used and the microbial load (Farooq and others, 1977). When treating *Candida parapsilosis* with ozone, a 4-log reduction resulted from an initial population of $1.4x10^5$ CFU/ml, though no reduction was observed from an initial population of $1.6x10^7$ CFU/ml (Farooq and others, 1977). Khadre and others (2001) gave guidelines for a uniform experimental design for stock culture experiments to establish standards for comparisons between studies. The guidelines enable one to establish results corresponding to the estimation of the number of ozone molecules sufficient to

inactivate a single bacterial cell (n_z) and to estimate the inactivation rate $\{\Delta (\log_{10}CFU/ml)/(\Delta \text{ time})\}$.

Table 2.3 lists numerous experiments that have been conducted on various food systems as well as specific bacteria. This information illustrates the imporatance of using a uniform experimental design as the experiment results in Table 2.3 are not directly comparable. Each experiment uses a different design, media, origin of bacteria and exposure times, therefore producing different log reductions. The studies using ozone as a disinfectant for seafood show varying results, which indicates that more research is required in this area. Kötters and others (1997) treated rockfish (*Sebastes* spp.) stored onboard the vessel in refrigerated seawater systems with recirculated ozonated water. The ozone enhanced detachment of surface bacteria and the shelf-life of the fish was extended 36 h. However, when the experiment was simulated in the laboratory, contrasting results were produced. The shelf-life of Atlantic cod (*Gadus morhua*) was not extended when exposed to ozonated ice, rinse water or chilled seawater (Ravesi and others, 1987). Silva and others (1998) observed that fresh scad (*Trachurus trachurus)* treated once with gaseous ozone for 60 min in a laboratory showed a one log reduction in total bacteria counts and in H_2S producing bacteria, from the fish skin samples. The second part of the study was carried out on board a fishing vessel in which the scad was treated initially with gaseous ozone for 60 min and

then once daily for 30 min, which showed a greater reduction in bacteria and longer bacterial lag times. A study on ozone treatments of jack mackeral and shimaaji (*Caranx metensi*) in 3% salt solution showed contrasting results (Haraguchi and others, 1969). Jack mackerel were treated every other day with 0.27-0.16 ppm ozone for 60 min, which resulted in a 2-day increase in shelf life. The ozone concentration was increased to 2.0-0.6 ppm in treating shimaaji every other day, which resulted in a 4-day increase in shelf-life. Chen and others (1987) found that treating frozen shrimp with ozone reduced *E. coli* counts by 98.5%. Aqueous ozone (2.9-4.8 ppm) with a contact time of 15-60 min was ineffective as a bacteriocidal for shrimp inoculated with nine different spoilage and pathogenic bacterial species (Chen and others, 1992). Out of the nine bacteria inoculated in the shrimp, *Flavobacterium aquatile* was reported to be the most sensitive as it had the largest log reduction of 0.89 after 60 min of treatment. Roe treated with a solution of 3% sodium, ozone (17.2 ppm) and 97.2 KBr/L showed a 2-day shelf-life increase delaying microbial growth during the first week of storage (Sakamoto and others, 1996).

Table 2.3. Application of ozone and effects on various microorganisms.

Microorganism	Origin of Bacteria	Medium	Log Reduction	Exposure Time (min)	Ozone mg/liter	Temp. (°C)	Ozone State	Reactor Type	Reference
Aerobic bacteria	broiler carcasses	broiler carcasses	0.66	45	3-4.5	7	aq	-	Sheldon and Brown, 1986
Aerobic bacteria	stock culture	inoculated shrimp meat	0.88	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
Aerobic bacteria	contaminate beef carcasses	contaminated beef carcasses	1.30	0.3-0.65	0.3-2.3	-	aq	continuous (spray)	Reagan and others, 1996
Aerobic bacteria	stock culture	inoculated whole shrimp	1.36	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
Aerobic bacteria	bee pollen	bee pollen	1.60	480	18	-	g	continuous	Yook and others, 1998
Aerobic bacteria	poultry chiller H_2O	poultry chiller H_2O	6.49	60	3-4.5	7	aq	-	Sheldon and Brown, 1986
Aeromonas liquefaciens	diseased fish	sterile water	3	1	1	-	aq	batch	Colberg and Lingg, 1978
A. salmonicida	stock culture	phosphate water	3	0.01	10	20	aq	continuous	Wedemeyer and Nelson, 1977
A. salmonicida	diseased fish	sterile water	8	1	1	-	aq	batch	Colberg and Lingg, 1978
Bacillus cereus	-	O_3 demand free H_2O	6	5	0.12	28	aq	-	Broadwater and others, 1973
B. megaterium	-	O_3 demand free H_2O	6	5	0.19	28	aq	-	Broadwater and others, 1973
Bacillus spp.	bee pollen	bee pollen	1.86	480	18	-	g	continuous	Yook and others, 1998
*Bacillus*spores	-	O_3 demand free H_2O	6	5	2.29	28	aq	-	Broadwater and others, 1973
Coliform bacteria	shredded lettuce	shredded lettuce	>3	3	0.15-0.2	-	aq	-	Montecalvo, 1998
Cryptosporidium parvum	stock culture	phosphate buffer H_2O	3	4.50	2	25	aq	batch (stirring)	Korich and others, 1990
Enterococcus seriolicida	yellowtail tuna	seawater	6	1	0.39	1	aq	batch (stirring)	Sugita and others, 1992
Escherichia coli	stock culture	phosphate buffer	0	5	600	22	aq	continuous (bubbling)	Komanapalli and Lau, 1996
E.coli	polluted waters	sterile distilled water	1	1.13	0.50	25	aq	continuous (bubbling)	Lezcano and others, 1999

Table 2.3. Application of ozone and effects on various microorganisms, Continued.

E.coli	stock culture	inoculated shrimp	1.99	15	1.40	5	aq	batch (bubbling)	Chen and others, 1992
E.coli	stock culture	phosphate buffer	2	30	600	22	aq	continuous (bubbling)	Komanapalli and Lau, 1996
E. coli	-	phosphate buffer	2	0.10	0.53	1	aq	batch	Fetner and Ingols, 1956
E. coli	stock culture	inoculated shrimp	2.19	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
E. coli	stock culture	waste water	2-2.5	-	-	-	aq	-	Arana and others, 1999
E. coli	-	raw wastewater	3	19	2.20	16	aq	continuous	Joret and others, 1982
E. coli	stock cuture	dried meat broth on stainless steel	3.10	240	2	22	g	continuous	Moore and others, 2000
E. coli	stock cuture	dried organic soy milk on stainless steel	3.30	240	2	22	g	continuous	Moorcand others, 2000
E.coli	stock culture	dried Ringer soln on stainless steel	3.40	240	2	22	g	continuous	Moore and others, 2000
E.coli	poultry chiller H_2O	poultry chiller H_2O	3.45	60	3-4.5	7	aq	-	Sheldon and Brown, 1986
E. coli	-	O_3 demand free H_2O	4	1.67	0.23-0.26	24	aq	continuous	Farooq and Akhlaque, 1983
E. coli	stock culture	0.8% saline	>5	9	0.50	25	aq	batch (bubbling)	Chen and others, 1992
E. coli	stock culture	phosphate buffer	5.51	1	0.19	-	aq	batch (stirring)	Finch and others, 1987
E. coli	-	O_3 demand free H_2O	6	5	0.19	28	aq	-	Broadwatcr and others, 1973
E. coli	stock culture	DI H_2O w/ 20 ppm SS	>6	1	0.19	19-21	aq	batch	Restaino and others, 1995
E. coli	stock culture	DI H_2O w/ BSA	6	5	0.19	19-21	aq	batch	Restaino and others, 1995
E. coli	stock culture	deionized water	6.20	1	0.19	19-21	aq	batch	Restaino and othcrs, 1995
E. coli O157:H7	stock culture	O_3 demand frce H_2O	3.80	0.50	1	25	aq	batch	Kim and Yousef, 2000
E. coli O157:H7	stock culturc	apples	3.70	3	36	22	aq	batch (bubbling)	Achen and Yousef, 2001
E. coli O157:H7	shredded lettuce	shredded lcttuce	4	3	0.30	-	aq	-	Montecalvo, 1998

Table 2.3. Application of ozone and effects on various microorganisms, Continued.

E. coli O157:H7	stock culture	phosphate buffer	6	30	10	-	aq	continuous (bubbling)	Byun and others, 1998
E. coli O157:H7	stock culture	TSA media	6	40	10	-	g	continuous	Byun and others, 1998
Flavobacterium aquatile	stock culture	inoculate shrimp	1.76	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
Heterotrophic bacteria	raw water (well & river)	raw water (well & river)	2.60	120-180	3	23	aq	continuous (bubbling)	Lee and Deinginger, 2000
Legionella pneumophila	-	distilled water	>4.5	20	0.32	24	aq	batch	Edelstein and others, 1982
Leuconostoc mesenteroides	stock culture	O_3 demand free H_2O	3.30	0.50	1.50	25	aq	batch	Kim and Yousef, 2000
L. mesenteroides	stock culture	O_3 demand free H_2O	7	0.50	4	25	aq	batch	Kim and Yousef, 2000
Listeria innocua	stock culture	dried meat broth on stainless steel	0.40	240	2	22	g	continuous	Moore and others, 2000
L. innocua	stock culture	dried organic soy milk stainless steel	0.80	240	2	22	g	continuous	Moore and others, 2000
L. innocua	stock culture	dried Ringer soln on stainless steel	3.30	240	2	22	g	continuous	Moore and others, 2000
L. monocytogenes	stock culture	deionized water	5.50	<0.5	0.19	19-21	aq	batch	Restaino and others, 1995
L. monocytogenes	stock culture	K- phosphate buffer	5.50	10	0.10	25	aq	continuous	Lee and others, 1998
L. monocytogenes	stock culture	O_3 demand free H_2O	5.70	0.50	0.80	25	aq	bacth	Kim and Yousef, 2000
L. monocytogenes	stock culture	DI H_2O w/ BSA	5.80	2	0.19	19-21	aq	batch	Restaino and others, 1995
L. monocytogenes	stock culture	DI H_2O w/ 20 ppm SS	6.80	<0.1	0.19	19-21	aq	batch	Restaino and others, 1995
Mycobacterium fortuitum	-	O_3 demand free H_2O	1	1.67	0.23-0.26	24	aq	continuous	Farooq and Akhlaque, 1983
Pasteurella piscicida	yellowtail tuna	seawater	6	1	0.17	25	aq	batch (stirring)	Sugita and others, 1992
Pseudomonas aeruginosa	polluted waters	sterile distilled H_2O	1	1.21	0.97	25	aq	continuous (bubbling)	Lezcano and others, 1999
P. aeruginosa	stock culture	inoculated shrimp	1.17	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
P. aeruginosa	stock culture	0.8% saline	>5	9	0.34	25	aq	batch (bubbling)	Chen and others, 1992
P. aeruginosa	stock culture	deionized water	5.80	1	0.19	19-21	aq	batch	Restaino and others, 1995

Table 2.3. Application of ozone and effects on various microorganisms, Continued.

P. fluorescens	stock culture	O_3 demand free H_2O	0.90	0.50	0.20	25	aq	batch	Kim and Yousef, 2000
P. fluorescens	shredded lettuce	shredded lettuce	<1	1	10	-	aq	batch	Kim and others, 1999
P. fluorescens	stock culture	inoculated shrimp	1.49	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
P. fluorescens	diseased fish	sterile water	3	1	1	-	aq	batch	Colberg and Lingg, 1978
P. fluorescens	stock culture	0.8% saline	>5	13	1.07	25	aq	batch (bubbling)	Chen and others, 1992
P. fluorescens	stock culture	O_3 demand free H_2O	5	0.50	1.20	25	aq	batch	Kim and Yousef, 2000
P. putida	stock culture	inoculated shrimp	1.10	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
P. putida	stock culture	0.8% saline	>5	11	0.78	25	aq	batch (bubbling)	Chen and others, 1992
psychrotrophic bacteria	broiler carcasses	broiler carcasses	0.20	45	3-4.5	7	aq	-	Sheldon and Brown, 1986
psychrotrophic bacteria	shredded lettuce	shredded lettuce	4.60	5	1.3mM	-	aq	batch (bubbling)	Kim and others, 1999
Salmonella spp.	broiler carcasses	boiler carcasses	0.72	45	3-4.5	7	aq	-	Sheldon and Brown, 1986
Salmonella spp.	poultry chiller H_2O	poultry chiller H_2O	2.10	60	3-4.5	7	aq	-	Sheldon and Brown, 1986
Salmonella typhimurium	polluted waters	sterile distilled water	1	1.69	1.04	25	aq	continuous (bubbling)	Lezcano and others, 1999
S. typhimurium	stock culture	inoculate shrimp	0.19	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
S. typhimurium	-	O_3 demand free H_2O	4.30	1.67	0.23-0.26	24	aq	continuous	Farooq and Akhlaque, 1983
S. typhimurium	stock culture	0.8% saline	>5	17	0.54	25	aq	batch (bubbling)	Chen and others, 1992
S. typhimurium	stock culture	deionized water	6	2	0.19	19-21	aq	batch	Restaino and others, 1995
S. typhimurium	stock culture	DI H_2O w/ 20ppm SS	6	2	0.19	19-21	aq	batch	Restaino and others, 1995
S. typhimurium	stock culture	DI H_2O w/ BSA	6	2	0.19	19-21	aq	batch	Restaino and othes, 1995
Serratia liquefaciens	stock culture	dried meat broth on stainless steel	2.80	240	2	22	g	continuous	Moore and others, 2000
S. liquefaciens	stock culture	dried organic soy milk stainless steel	1.50	240	2	22	g	continuous	Moore and others, 2000
S. liquefaciens	stock culture	dried Ringer soln on stainless steel	3.50	240	2	22	g	continuous	Moore and others, 2000

Table 2.3. Application of ozone and effects on various microorganisms, Continued.

Shigella	shredded lettuce	shredded lettuce	>3	3	0.15	-	aq	-	Montecalvo, 1998
Staphylococcus aureus	stock culture	dried meat broth on stainless steel	0.80	240	2	22	g	continuous	Moore and others, 2000
S. aureus	stock culture	inoculated shrimp	1.73	60	1.40	5	aq	batch (bubbling)	Chen and others, 1992
S. aureus	stock culture	dried organic soy milk stainless steel	2.80	240	2	22	g	continuous	Moore and others, 2000
S. aureus	stock culture	dried Ringer soln on stainless steel	4.30	240	2	22	g	continuous	Moore and others, 2000
S. aureus	stock culture	deionized water	5	2	0.19	19-21	aq	batch	Restaino and others, 1995
S. aureus	stock culture	DI H_2O w/ 20 ppm SS	5	2	0.19	19-21	aq	batch	Restaino and others, 1995
S. aureus	stock culture	DI H_2O w/ BSA	5	5	0.19	19-21	aq	batch	Restaino and others, 1995
S. aureus	stock culture	0.8% saline	>5	5	0.30	25	aq	batch (bubbling)	Chen and others, 1992
Total bacteria count	spoiled poultry	distilled water	7.08	4	19	25	aq	batch	Yang and Chen, 1979
Total bacteria count	spoiled poultery meat	Ringer solution	1.50	3	19	25	aq	batch	Yang and Chen, 1979
Total bacteria count	spoiled poultry meat	5% NaCl	2	5	2.48	2	aq	batch	Yang and Chen, 1979
Total bacteria count	spoiled poultry meat	egg albumin	0.15	7	2.48	-	aq	batch	Yang and Chen, 1979
Total bacteria count	fresh poultry meat	distilled water	3.63	10	37.70	2	aq	batch	Yang and Chen, 1979
Vibrio anguillarum	yellowtail tuna	seawater	6	1	0.23	25	aq	batch (stirring)	Sugita and others, 1992
Yersinia enterocolitica	stock culture	deionized water	5.80	5.80	0.19	19-21	aq	batch	Restaino and others, 1995

(aq): aqueous ozone, (g): gaseous ozone; Reactor type: treatment type (flow used to treat the samples).

OZONE VERSES CHLORINE

Though ozone technology has existed since the 1800s, chlorine has been the disinfectant of choice since World War I (Brink and others, 1991; Khadre and others, 2001). In the past, chlorination has been an inexpensive way to disinfect, though with new studies and understanding of ozone, cost is no longer the determining factor (Forsythe and Waldroup, 1994). Ozone has a high oxidation reduction potential of 2.076 V, which is much higher than chlorine at 1.36 V and hypochlorite at 0.954 V (Bablon and others, 1991). Ozone is an efficient oxidizer with a solubility ratio of 0.64 gas/water at 0°C as it takes a larger amount of chlorine with a higher solubility ratio of 4.54 to produce similar sanitizing results to that of ozone (Masschelein, 1982). Ozone is also growing in acceptance and use due to the findings of some negative attributes of chlorine (Brink and others, 1991). It was discovered in 1974 that when chlorine was used as a water disinfectant it could react with organic matter to form chemical by-products (Tibbetts, 1995).

Residual chlorine has some significant disadvantages. Excessive chlorine residues >0.1 mg/L could be toxic in areas of critical biological significance (Brungs, 1973; Muela and others, 1998; Richardson, 1998). It may also be necessary to provide dechlorination facilities (Collins and Deaner, 1973). Santorum and others (1999) reported after ozone and chlorine disinfections of wastewater, chlorinated water toxicity was higher when compared to ozonated wastewater

toxicity. Moreover, after 2 h of disinfecting, no toxicity could be detected from ozonated samples. Ozone does not have any toxic residues (Achen and Yousef, 2001).

Alternative water treatment methods such as ozonation (Morris, 1971; Graham, 1997), have received increased attention with the discovery that chlorine can form halogenated organic compounds (Rook, 1974; Jolley, 1975; Bull, 1982; Junli and others, 1997) such as trihalomethane compounds (THMs) in water. THMs in drinking water are formed by the reaction of free chlorine (HOCl, OCl^-) with soluble organic compounds (Kim and Yousef, 1999). Chlorination is known to produce chemical compounds that cause cancer (Wei and others, 1985; Kraybill, 1987; Tyrrell and others, 1995; Richardson, 1998) the most significant being chloroform (Cheh and others, 1980; Junli and others, 1987a, b, c), bromodichloromethane and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (Richardson, 1998). Chronic exposure to chlorine or chlorine by-products could cause liver, kidney, heart, and neurological damage including effects to unborn children (Tibbetts, 1995).

Several studies have compared reduction times for microorganisms in the presence of ozone and chlorine. Kessel and others (1943) reported that free ozone residues from 0.05 to 0.45 mg/L were adequate to inactivate poliovirus within

2 min, while free chlorine residues of 0.5 to 1.0 mg/L at pH 6.0 were required for 1.5 to 2.0 h for a similar degree of inhibition. Korich and others (1990) investigated oocyst viability of *Cryptosporidium parvum* and reported >90% inactivation with 1.0 mg/L of ozone for 5 min. At 80 mg/L of chlorine for 90 min, a 90% inactivation of oocysts in drinking water was reported. Santorum and others (1999) reported both ozone and chlorine produced similar microbial reductions after treating *E. coli* suspended in wastewater. They also observed that chlorinated cells tended to aggregate raising the question, to what degree of interference do aggregated cells cause in conventional colony counting methods?

FUTURE OF OZONE

More research with ozone is needed to understand its full potential with food systems. Now that the U.S. government has accepted ozone as a GRAS compound in food processing, a broader range of food studies can be initiated. One area where ozone applications in seafood processing may be enhanced is the introduction of a preliminary rinse stage before ozone treatment. Ozone reacts rapidly with organic material such as mud, slime layer and blood. A pre-rinse would alleviate some of the initial demand on the ozone and could increase the disinfectant power significantly. Some studies add ozone into the preliminary washing stage and then combine the two. This effectively increases the demand of

the ozone and decreases its disinfecting power. It is also suggested that the combination of two or more disinfectants used together or in a series may enhance ozone efficacy. Ozone in combination with hydrogen peroxide proved more effective than ozone alone in destroying toxins produced by blue-green algae (Rositano and others, 1998). Mitsuda and others (1990) studied the sterilization abilities of both ozone and carbon dioxide as individual gases and as a mixture of gases. The most effective sterilization was produced from the mixture of the two gases. Effective sterilization resulted from a nanosecond electron beam and the ozone that is produced (Kotov and Sokovnin, 2000). A greater reduction in bacterial load was achieved when ozone and UV photons were used together to kill bacteria in poultry processing water (Diaz and others, 2001). Ozone has also been evaluated for medical disinfectant purposes. Ozone has proven to be a superior disinfectant in dental treatment units (Filippi, 1997). Ozonated water can be used in dental, oral and maxillomandibular surgery.

With the FDA acceptance of ozone as GRAS, the potential uses and technology development are expanding for broader opportunities in disinfecting surface areas, food and food processing, water and storage areas to a multitude of medical uses. It is suggested that further in-depth experiments address: 1) the process of how ozone disinfects at the cellular and molecular levels, 2) ozone efficacy on various substrates within the treatment system, and 3) the disinfection pathway of ozone in combination with other, accepted methods and chemicals.

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CHAPTER 3: EFFECT OF OZONE TREATMENT ON OYSTERS, ROE AND SALMON FILLETS

ABSTRACT

Pacific oysters, Alaskan pink salmon roe and chum salmon fillets were treated with aqueous ozone and stored at 4°C over different time periods. Oysters and roe were treated with 0.5-1.0 ppm ozone at 5°C and 15°C for 30 s, 2 and 4 min and fillets were treated with 1.3 ppm ozone at 9°C for 2 min. The microbial quality of the samples was analyzed by conducting pyschrotrophic and coliform plate counts. Oxidative rancidity, pH and moisture were measured during the shelf-life study to determine the effects of ozone on the quality of the seafood. Although there were some minor decreases in microbial growth associated with ozone treatments, no increase in shelf-life was observed.

INTRODUCTION

Seafood quality can decrease from the point of harvest and throughout the processing and distribution chain. Currently, there are no effective quality handling specifications for fish and oyster harvesting boats. The seafood processor becomes responsible for fish and shellfish that are harvested and off-loaded at the plant. Incoming fish and oysters have varying microbial loads due to storage time, temperature and other variable factors prior to delivery to the processors (Shewan, 1949; Thompson and Vanderzant, 1976). The processing facilities must either decrease bacterial numbers or stop bacterial growth. Lowering the temperature of incoming seafood by using ice or slush totes (mixture of ice and water) is ideal for slowing down the microbial growth rate. To extend shelf-life, a disinfection process could also be applied to decrease the microbial load and subsequent growth on seafoods that are minimally processed.

Oysters

The Pacific oyster (*Crassostrea gigas*) is a West Coast shellfish species that is commonly marketed as a fresh, shucked product (Hoff and others, 1967a). When

harvested, live shellfish contain large numbers of microorganisms that remain in the shellfish through all subsequent phases of handling and marketing (Hoff and others, 1967b). Decreasing spoilage and increasing shelf-life are imperative for oysters sold as a fresh refrigerated product. The initial microbial load of oysters, at the point of harvest, has a large variation and the aerobic plate counts can range from $2.3x10^4$/g to $3.0x10^7$/g (Vanderzant and Thompson, 1973). Levels of bacteria found in oysters harvested from a number of locations throughout New South Wales ranged from $9.0x10^3$/g to $6.5x10^4$/g (Son and Fleet, 1980). Sanitary conditions must be maintained in oyster processing plants to prevent or delay bacterial growth increases during storage.

Depuration processes used in oyster processing may decrease but not prevent microbial spoilage. Depuration can decrease the amount of pathogenic bacteria that reside inside oysters, but the amount of spoilage bacteria is usually unchanged. During depuration, total plate counts can be decreased 10-fold, but rarely fall below 10^4 cells/g of oyster (Son and Fleet, 1980). Despite depuration, oysters will maintain an indigenous microbial flora level of 10^4 coloney forming units per gram (CFU/g) (Soundess and Fleet, 1979). It has been suggested that processing facilities focus on preventing spoilage by decreasing the surface bacteria of the oyster rather than the internal bacteria for fresh, shucked oysters. Spoilage can set in quickly after harvest due to an increase in the microbial load, holding time and temperature. However, oysters that meet microbial standards at processing

facilities, and maintained at refrigeration temperatures thereafter, could still have well over 10^6 CFU/g by the time the product reaches the consumer. Therefore, decreasing the initial microbial load of oysters is vital to improving the consumer marketing and consumption of raw oysters. A microbial load exceeding 6 logs is considered spoiled (Andrews and others, 2000).

Roe

Salmon roe is a salt-brined, cured product typically processed in two forms. Sujiko is a heavily salted product with eggs still in skeins and ikura is a less salted product that is sold as individual eggs. Sujiko traditionally exhibits a salt content of 7-10% and ikura has an average salt content of 2.5-4%. The roe of coho, sockeye and pink salmon is used to make sujiko, whereas, ikura is characteristically made from chum and pink salmon roe. Ikura requires a greater amount of processing (Fig. 3.1) than sujiko roe (Himelbloom and Crapo, 1998). Only the ikura form was included in this study.

Ikura is more popular than sujiko and has a higher market value (Fig. 3.2). Japan is the primary importer of both sujiko and ikura roe. Salmon roe is a ready-to-eat food. Currently, there are no USA regulations governing the microbial limits

for exported seafoods. Regulations are dependent upon the import country and companies to provide individual standards. After the 1998 occurrence of *E. coli* O157:H7 contamination in Japanese roe (Moir, 1998), the import standards from Japan for U.S. roe processors became more stringent.

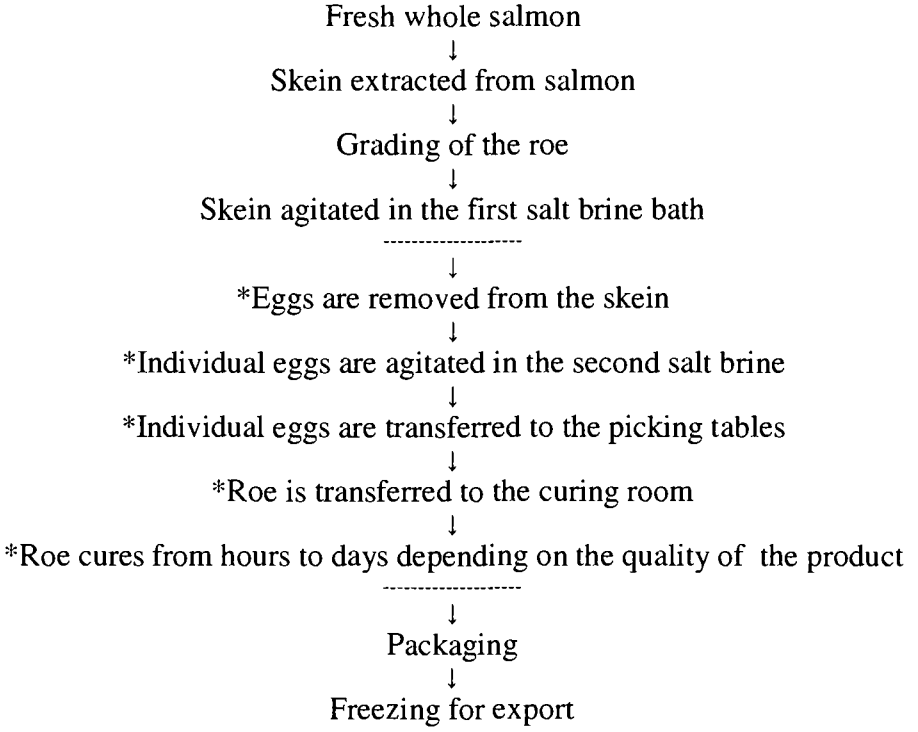
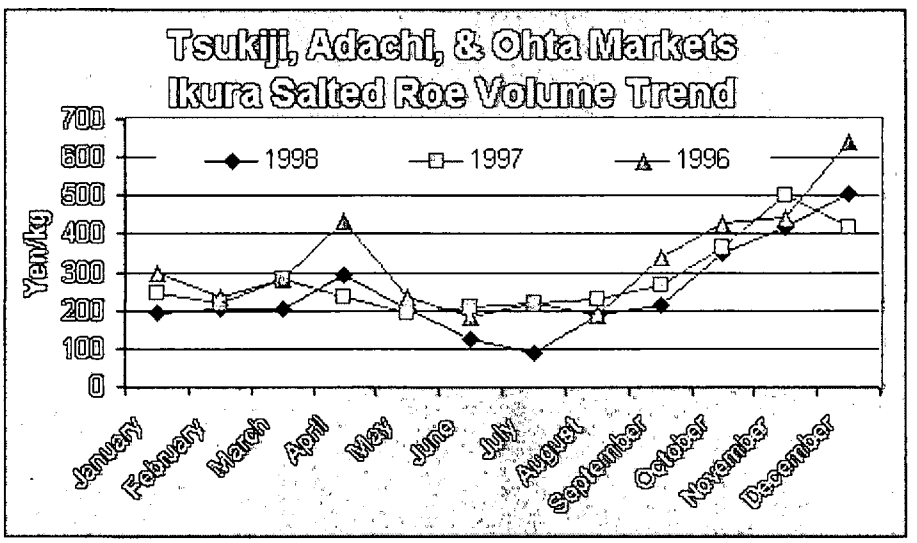


Figure 3.1: Roe processing. *Ikura processing only
After the skein is extracted from the salmon it is graded based on quality and age of the roe. Roe processing begins by bathing the skein in salt brine, then removing the indivual eggs from the skein by hand and bathing the individual eggs in a second salt brine. The eggs are then transferred to tables were the brine drains off the eggs and any leftover skein or fish parts are removed. From the picking tables the roe is cured in a room set at a specific temperature and humidity for up to 36 h. After curing the roe is packed into containers for export and transferred to frozen storage at < -20°C.

In the summer of 1998, roe from a processing plant in Hokkaido, Japan was recalled following investigations of several cases of food poisoning (Shimbun, 1998). The processing facility was closed soon after investigations found that the company stored and re-froze over 5 tons of roe that was returned due to its high bacterial load. Analyses using pulsed field gel electrophoresis, phage typing and epidemiological data showed 62 total cases of food poisoning from *E. coli* O157:H7 (Terajima and others, 1999; Makino and others, 2000). These cases in Japan indirectly threatened the Alaskan roe market. After extensive investigations, the Hokkaido government authorized lifting the ban on U.S. roe sales, once again allowing the importation of Alaskan roe into Japan (Lynn, 1998). Several exporters began searching for new technologies, such as ozone, to decrease or eliminate bacterial contamination in roe products.



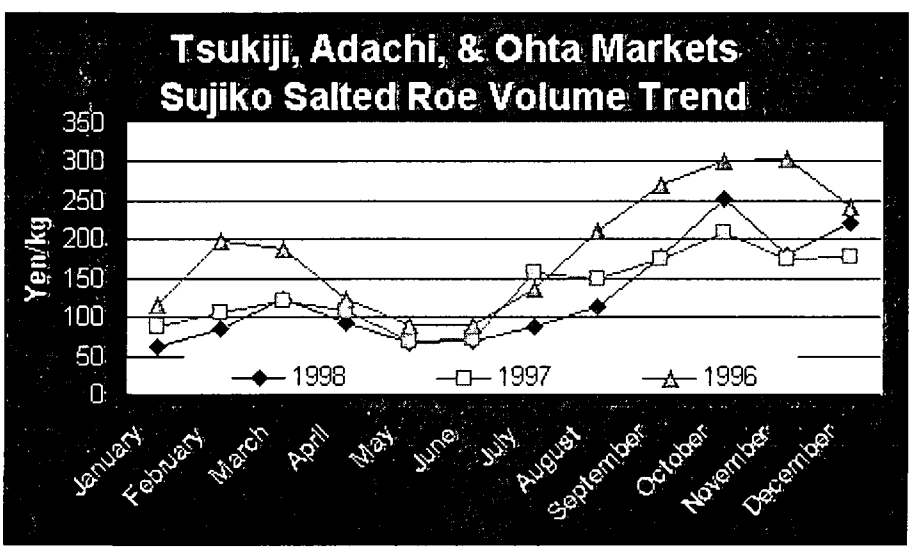


Figure 3.2. Revenue generated by ikura and sujiko (Alaska Division of International Trade and Market Development). http://www.madeinalaska.org/trade/roevol/12.htm

Fillets

Salmon is critical to the Alaskan seafood economy and makes up 14% ($98 million) of all exported seafoods (Alaska Division of International Trade and Market Development, 1998). Most species of fish are more perishable than other types of meats due to the chemical breakdown in fish muscle resulting in a high final (post-mortem) pH. High pH values have been linked with increased microbial growth rates.

Fresh salmon can have relatively low bacteria counts, depending on the harvesting conditions (Shewan, 1949). Two studies on salmon reported counts of $7.9x10^3$ CFU/cm^2 and $4.0x10^2$ CFU/g, upon delivery to processing facilities (Himelbloom and others, 1991; Crapo and Himelbloom, 1999). The microbial load can increase from processing by using insufficiently cleaned equipment (Himelbloom and others, 1991). After the filleting process, microbial counts have increased to 10^4-10^7/g, with 50% of the samples having counts greater than 10^6/g (Shewan, 1949). After the fish are handled and processed, the microbial count of each individual fish fillet is unknown. One potential way to alleviate this problem is by treating each fillet using a uniform decontamination process.

Ozone Studies

Shelf-life studies for seafood treated with ozone have shown varying results. Haraguchi and others (1969) reported a 4-day shelf-life increase in scad (*Trachurus trachurus*) and mackerel (*Caranx mertensi*) with the use of 30-60 min aqueous ozone washes once every other day. Kötters and others (1997) investigated the reproducibility of extending the shelf-life of rockfish (*Sebastes* spp.) by using aqueous ozone intermittently in the boat hold. Two experiments, were conducted, one on a fishing vessel equipped with an ozone generator, and the second being a laboratory simulation. The simulation experiment could not replicate the vessel test results. Daily exposure to gaseous ozone for 30 min with an initial 60 min exposure decreased the total viable count by 1 log in fresh scad (Silva and others, 1998). No shelf-life extension was found using either aqueous ozone treatments for 20 min per day or storage on ozonated ice for fresh Atlantic cod (*Gadus morhua*) (Ravesi and others, 1987).

There are few reports on the use of salmon roe with ozone. One study reported a 3-day increase in the shelf-life of processed roe using aqueous ozone and showed that the length of treatment time (0.5-5 min) resulted in a microbial decrease of <0.5 log (Sakamoto and others, 1996). No reports of using ozone to reduce the microbial count in oysters were found. Personal communication and visits to several oyster processing facilities confirmed that the industry was

interested in using ozone and some companies were exploring the use of ozone on their own.

The purpose of this study was to determine whether ozone would effectively lower microbial growth and prove beneficial to the seafood industry.

MATERIALS AND METHODS

Each experiment was done independently and methods were modified for each type of seafood tested. The oyster and salmon roe experiments were carried out at two different temperatures and undertaken at the Oregon State University Seafood Laboratory (OSU-SFL) in Astoria, Oregon. The use of ozone as a disinfectant on fish fillets was conducted at the Fishery Industrial Technology Center (FITC) in Kodiak, Alaska.

Oysters

Pacific oysters were obtained in May, 2000 from Goosepoint Oyster Co., Bay Center, WA. The oysters were harvested from oyster beds in Willapa Bay, WA, shucked and packed (unwashed) in their own juices in 5-lb plastic pails. The shucked meats were delivered on the same day to the OSU-SFL, in a refrigerated

van and kept at 2-4°C. Each five-pound pail contained an average of 130 small oysters. Prior to treatment, meats from 12-13 small oysters were weighed and packaged in Ziplock® bags. Each bag of oyster meats weighed an average of 227.8±6.4 g. Tests were run on the same day as delivery and the oysters were kept on ice until treated.

Roe

Unprocessed Alaskan pink salmon (*Oncorhynchus gorbuscha*) skeins were obtained from Wards Cove Packing Company, Excursion Inlet, Alaska. The skeins were harvested, packaged and frozen at the processing facility. The frozen skeins were delivered in 50-lb fish boxes to OSU-SFL, Astoria, OR and stored in a freezer (-20°C) until 24 h before treatment. Roe was thawed under refrigeration at 5-6°C for 24 h and kept on ice until tested.

Fillets

Chum salmon (*Oncorhynchus keta*) were harvested from Alaskan waters and headed and gutted (H&G) at the Excursion Inlet facility. The frozen fish were delivered to FITC, Kodiak, AK in 50-lb fish boxes on September 2, 2000 and stored at -40°C. Fish were thawed for 24 h before treatment. During the first 8-10 h

the fish were thawed in the pilot plant (21°C) in covered bins. For the remaining thaw time, the fish were stored in the refrigerator (4°C). All fish were filleted and weighed before each treatment.

Ozone Treatment

Aqueous ozone was produced using a Zing™ Ozone System (Net Systems, Bainbridge Island, WA). Oxygen was filtered from the surrounding air using an oxygen concentrator then passed through the ozone generator (Fig. 3.3). Ozone and water were mixed together using a Vortex™ mixing tank (Net Systems, Bainbridge Island, WA). Ozone concentrations were measured colorimetrically using a DR/850 colorimeter (Hach, Loveland, CO) employing the indigo method (Grunwell and others, 1983).

The oyster and roe experiments occurred in the OSU-SFL pilot plant. An ozone concentration of 0.5 ppm was maintained by setting the volumetric flow rate of water at 10 gpm under 22 psi at a rate of 5.5 ft^3 O_2 /h. To generate 1.0 ppm of ozone, oxygen was increased to 9.5 ft^3/h. The fillet experiments took place at the FITC. An ozone concentration in the experimental system of approximately 1.2-1.5 ppm was maintained by setting the volumetric flow rate of water at 10 gpm under 22 psi and a rate of 20 ft^3 O_2/ h.

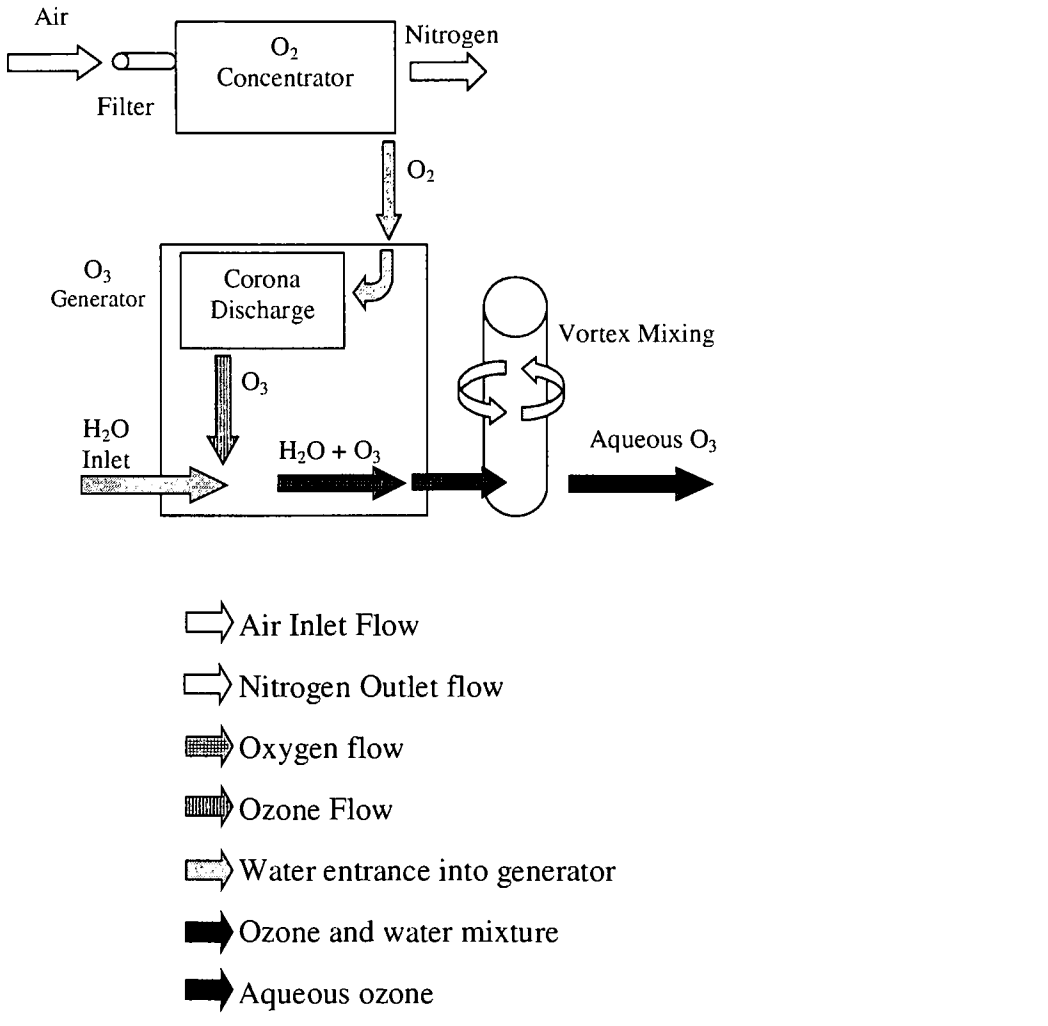


Figure 3.3. Air to aqueous ozone scheme.
Ozone can be made from passing air through a series of filters and exposing it to a high voltage alternating current called a corona discharge. The nitrogen must first be filtered out of the air to create a high percent of pure oxygen. The filtered oxygen is then forced through the corona and the gas exiting the corona is ozone. Through vigorous vortex mixing of water and ozone, aqueous ozone is produced (Ewell, 1950; Kim and others, 1999).

Treatment System

Oysters and Roe

An industrial size stainless steel crab steamer kettle (200 L) was converted into a closed system wash tank (Fig. 3.4A, B). In creating a closed system, the lip of the steamer and the rim of the lid were sealed tightly with 0.95 cm x 0.48 cm foam weatherstripping (Frost King, Thermwell Products, Mahwah, NJ). The lid of the steamer was stainless steel and hinged making one smaller and one larger piece. The larger sector of the lid was continually clamped down (except for cleaning) and the smaller portion of the lid was used to access the samples inside the kettle. There was a single inlet and outflow in the system that was controlled manually. Both non-ozonated and ozonated water used the same inlet and outflow. A hose aided in creating a closed system and was used to transfer water from the ozone generator to the crab steamer. A hole was cut into the lid of the crab cooker and polyvinyl chloride (PVC) piping was plumbed into the hole. The hose was the connection between the ozone generator and the pipe in the lid. The closed system had dual functions, to protect workers from being exposed to ozone gas and to prevent ozone from leaking into the pilot plant atmosphere.

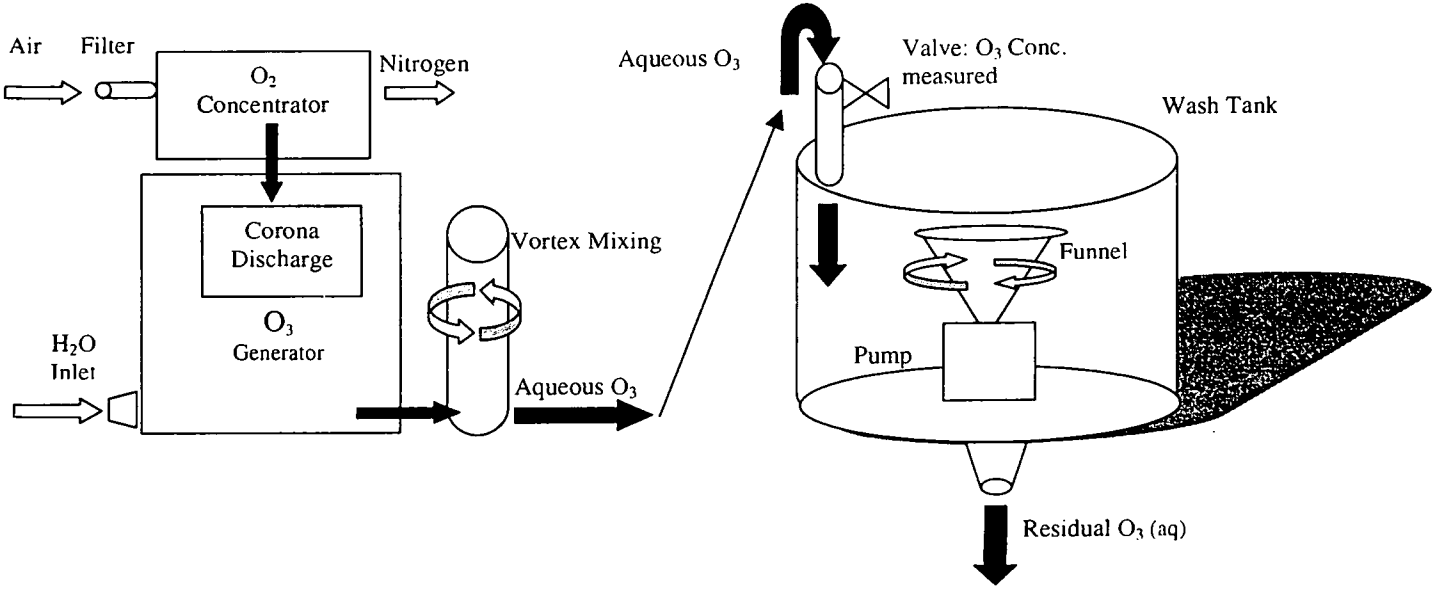


Figure 3.4.A. Ozone wash system. After the aqueous ozone is generated from incoming air and water from the pilot plant, it travels through a hose from the vortex-mixing chamber to the wash tank. A check valve plumbed into the receiving pipe, in the lid of the wash tank was used in monitoring the incoming ozone concentrations. The water exited the bottom tank after each wash, where the residual ozone was sampled for analysis.

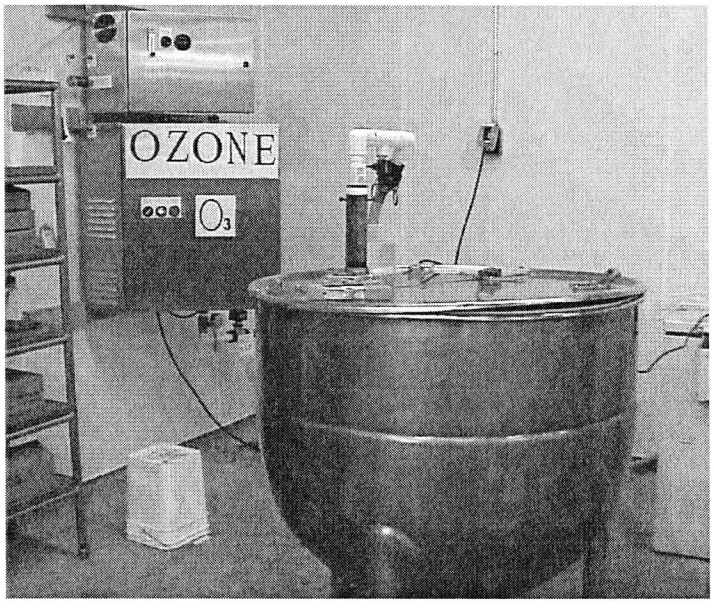


Figure 3.4.B. Ozone wash system used in the oyster and salmon experiments.

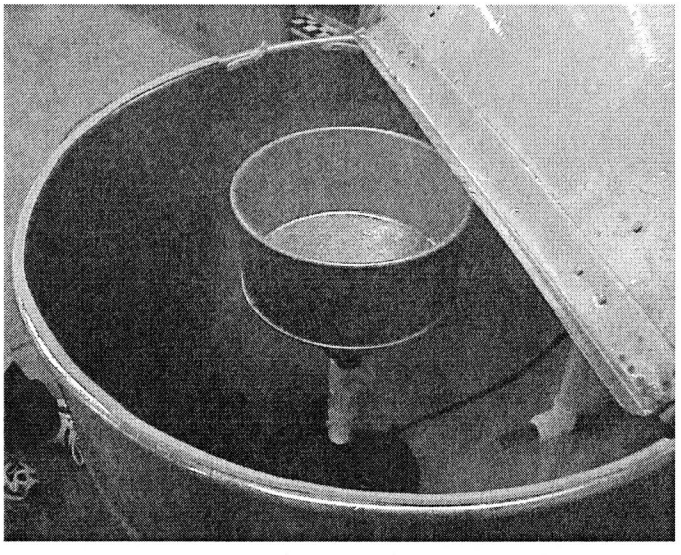


Figure 3.5. Funnel used to house oysters and roe for treatment application.

All samples were placed into a container inside the modified crab steamer (Fig. 3.5). A water agitator was fabricated from a sump pump and an industrial aluminum funnel that was plumbed together with an adjustable PVC pressure handle between the pump and the funnel. The sump pump suctioned water from the bottom of the crab steamer into the funnel, which created a gentle circulating agitation action on the oysters and roe.

When the crab cooker was filled 2.5-5.1 cm from the top of the steamer, the water inlet valve was shut off, allowing each batch of samples to be treated uniformly with approximately 200 L of ozonated or non-ozonated water. Oysters or roe were emptied into the inner funnel one bag at a time. A screen was placed over the top of the funnel holding the oyster meats and roe within and the crab cooker lid was shut and clamped down. Timing of the process began when the sump pump was started. At the end of each treatment, the pump was turned off and the screen removed. A small hand-held strainer was used to remove the oyster meats. Roe was removed from the funnel using latex gloves. Oysters and roe were transferred directly into pre-labeled Ziplock® bags and immediately iced until analyzed. All equipment used was washed with pressurized water between each treatment.

Oyster and roe tests were performed at 5°C and 15°C. Experiments at 15°C used tap water alone or combined with aqueous ozone in the closed wash system (Fig. 3.4A, B). A metal thermometer recorded the water temperature that exited the wash tank after each treatment at 5°C and 15°C. Tests run at 5°C used iced tap

water alone or combined with gaseous ozone in the closed wash system. The water entered a tote continuously supplied with flake ice to maintain the water temperature at 5°C. The fresh water flake ice was made and donated by a local seafood processor. Chilled water entered the ozone generator by a connecting hose from the tote containing a mixture of flake ice and water. The hose was pulling water from the bottom of the tote to ensure only chilled water entered the ozone generator. The water temperature in the totes was continually monitored using digital temperature probes. The ozonated and non-ozonated chilled water was pumped into the ozone generator at 6.68 gpm.

Treatment parameters were: no wash (control), water wash (WW), 0.5 ppm ozonated water (0.5 ppm O_3), and 1.0 ppm ozonated water (1.0 ppm O_3). Treatments were applied to one sample at a time, for a period of 30 s, 2 min and 4 min. The water wash treatment technique was the same as the ozone wash treatment only no ozone was generated. Each treatment was performed in triplicate.

Fillets

Fish fillets were treated in a 160 L stainless steel crab steamer converted into a closed system wash tank to maintain control of the ozone. The treatment system was similar to the one previously described for oysters and roe only differing in the sample substrate, the tank lid material and the inner container of the

tank. The lid was composed of two pieces of plexiglass hinged together with duct tape. The larger portion of the lid was clamped down to the tank while the small piece was free to open and close. The inner container was a cage fabricated from two shallow, rectangular, plastic baskets connected together with plastic twist ties. A hole was cut in the top of the basket cage and the hose from the ozone generator was fitted tightly into the hole, forcing the incoming stream of water directly over the test fillet. The pilot plant hood was kept on at all times during the ozonation process to vent ozone from the processing area.

Fillet tests were run by placing one fillet into the submerged cage when the tank was full of water, approximately 2.5-5.1 cm from the top of the tank. The fillet was positioned directly under the flow of water from the hose. Treatment times began when the fillets were placed into the cage and the lid to the tank was clamped closed. The fillet was removed from the cage using latex gloves, drained for 30 s, placed in freezer grade Zip-lock® bags and kept on ice until all the treatments were completed.

The wash system for the fillets differed from oyster and roe experiments. The fillets were exposed to a continuous flow system while the oysters and roe were exposed to a batch ozonation system. In the continuous flow system, the tank water in-flow and out-flow were continuous and equal. The equal flow rates resulted in a full tank at all times with minimal space between the water level and

the lid. A residual ozone level (0.8-1.15 ppm) was maintained for those samples treated with ozonated water. After each treatment, the tank was washed, scrubbed and disinfected with PACE T-20-XX (Cleaner-Disinfectant-Detergent-Sanitizer, Nalso Diversified Technologies, Inc., Chargrin Falls, OH).

Treatment parameters for fish fillets were: no wash (control), water wash (WW), and 1.3 ppm ozonated water (1.3 ppm O_3). Treatments were applied to one fillet at a time in 2 min increments. Experiments were conducted in duplicate. Tests were run at water temperatures of 8-9°C.

Shelf-life Analysis

Oyster Preparation

After treatments, two to three oyster meats from each treatment type, depending on size, were placed in sterile jars for the shelf-life studies. Jars were filled with 15°C tap water (mimicking most industrial oyster processors) or 5°C water and refrigerated at 4°C. The oyster meats treated at 15°C were analyzed at days 0, 4, 8, 12, 18 and 21 and samples treated at 5°C were analyzed at days 0, 3, 6, 9, 15 and 21 and oyster meats treated. All jarred oysters were stored at 4°C.

Roe Preparation

After ozone treatments were finished, the skeins were stored in individual Zip-Lock® bags and stored at 4°C. The skeins were analyzed at days 0-4 for the experiment at 15°C and at days 0, 2, 3 and 4 for the experiment at 5°C. For skeins treated with 1.0 ppm ozone at 5°C, only one contact time of 30 s was sampled as the roe quality began to diminish in the samples thereafter.

Fillet Preparation

Treatments were carried out on five separate days. The sample experimental design was the same for each treatment day, using 6 fillets, 2 controls, 2 treated with ozonated water and 2 treated with non-ozonated water. Only one group of fillets was used for each testing day. All fillets were stored at 4°C in individual Zip-Lock® bags. The fillets were tested on days 0, 2, 5, 7, and 10.

Microbial Analysis

Oysters and Roe

Oyster meat samples were drained and aseptically transferred from storage jars to sterile blending jars and blended for approximately 1 min. Roe samples were removed from the storage Zip-Lock® bags and transferred to a sterile stomacher bag (Whirl-pak, Nasco, Fort Atkinson, WI) and stomached (Sheldon and Brown, 1986) for 1 min. For psychrotrophic plate counts (PC) and coliform counts of oysters and roe, an 11 g aliquot of blended oyster or homogenized roe was aseptically weighed into a sterile stomacher bag containing 99 ml of 0.1% sterile peptone water and mixed using a masticator (1 min, 8 strokes/s, IUL Instruments, Barcelona, Spain) for 1 min. Subsequent serial dilutions were made using 99 ml of 0.1% peptone together with 1 ml diluent from the first dilution and agitated in bottles for 30 s.

Pyschrotrophic plate counts (PC) were carried out for oysters using either 1 ml or 0.1 ml aseptically transferred to a petri dish in the 15°C experiments and 1 ml was transferred to Aerobic Count (AC) Plate petrifilm (3M Microbiology Products, St Paul, MN) for the 5°C experiments. For roe experiments at 15°C and 15°C, petrifilm was used for both PC and coliform counts using a 1:10 dilution. In both the oyster and roe experiments, E. coli/Coliform Count (EC) Plate petrifilm was

used to obtain coliform colony counts. Petrifilm has a coloney detection minimum of 10 CFU/g as all samples were plated from a minimum of 10^{-1} dilution.

All dilutions were plated out in triplicate using either the pour plate (Restaino and others, 1995) or petrifilm (Priego and others, 2000) methods. Total plate count (TPC) agar (Chen and others, 1992) was used in the pour plate method. Petrifilm and petri plates were incubated at 25°C and colonies were enumerated at 48 to 72 h (Himelbloom and Crapo, 1998) The AC petrifilm incubation temperature was modified to 25°C to promote pyschrotrophic bacterial growth. Coliform colonies were enumerated after 48 h at 35°C (Priego and others, 2000).

Fillets

The 25 g aliquots of fillet were taken from the first cm of outer muscle layer, since bacterial spoilage happens primarily at the surface (Sikorski, 1990). The aliquots were aseptically weighed into a sterile stomacher bag containing 225 ml of 0.1% sterile peptone water and homogenized in the stomacher for 1 min. Subsequent serial 1:10 dilutions of homogenized fillet were made using 0.1% peptone. The dilutions were vortexed (Mini Vortexer MV 1, VWR Scientific Products, IKA Works, Wilmington, NC) in 25 ml test tubes for 30 s and 1 ml was transferred onto AC and EC petrifilm. Total coliform analyses were carried out in

parallel with PC dilutions. Psychrotrophic and colifrom plate counts were incubated and enumerated as described above. Microbial enumerations were carried out in triplicate.

Moisture Analysis

Duplicate 10-12 g aliquots of the blended oyster meat and homogenized roe samples and triplicate aliquots of fillet samples were weighed in aluminum pans for moisture analysis. The weighed aliquots were transferred directly to a drying oven set at 105°C and dried for a minimum of 24 h. Dried samples were reweighed and the percent moisture was calculated (method 950.46. AOAC, 1996).

Oxidative Rancidity Analysis

The 2-thiobarbituric acid oxidative rancidity (TBA) test (Sinnhuber, 1958) was used. Aliquots from the blended oyster meats and homogenized roe samples were transferred into prelabeled, conical 50 ml plastic tubes and held on ice. The tubes were flushed with nitrogen gas and stored frozen (-51°C). When needed, the samples were thawed in a refrigerator and analyzed. A Sorvall (Wilmington, DE)

RC-5B refrigerated superspeed centrifuge was used for centrifuging the samples (10 min, 2000 rpm) prior to analysis.

In the TBA calculation for oysters, using the gross weight was not a precise measurement due to the large variation in moisture. Dry weight determination of each oyster sample gave a more precise calculation of TBA, since the moisture content is known to affect the rate of rancidity in some foods (Rhee and others, 1983). The weight of each oyster aliquot used for TBA analysis was corrected on a dry weight basis (μg malonaldehyde (MDA)/g dry wt sample).

pH Analysis

Oysters and Roe

Aliquots (1 ml) of the blended oyster meat and homogenized roe were placed in 50 ml conical plastic tubes on ice and 9 ml of de-ionized water were added. The mixture was vortexed (VWR Scientific Vortex-2 Genie, Portland OR) for 30 s and pH was measured using a Corning 240 pH meter (Corning Glass Works, Medfield, MA).

Fillets

Aliquots (1 g) of fillet were weighed out into stomacher bags and stored at 4°C for 2-5 h for further analysis. Nine ml of deionized water were added to the 1 g aliquots and stomached for 30 s. The pH was measured using a Corning 240 pH meter. The analyses were carried out in triplicate.

Statistical Analysis

Excel (Microsoft Millennium Ed., Windows 2000, Gateway, North Sioux City, SD) was used for calculation of the standard deviation, T-test and the regression analysis.

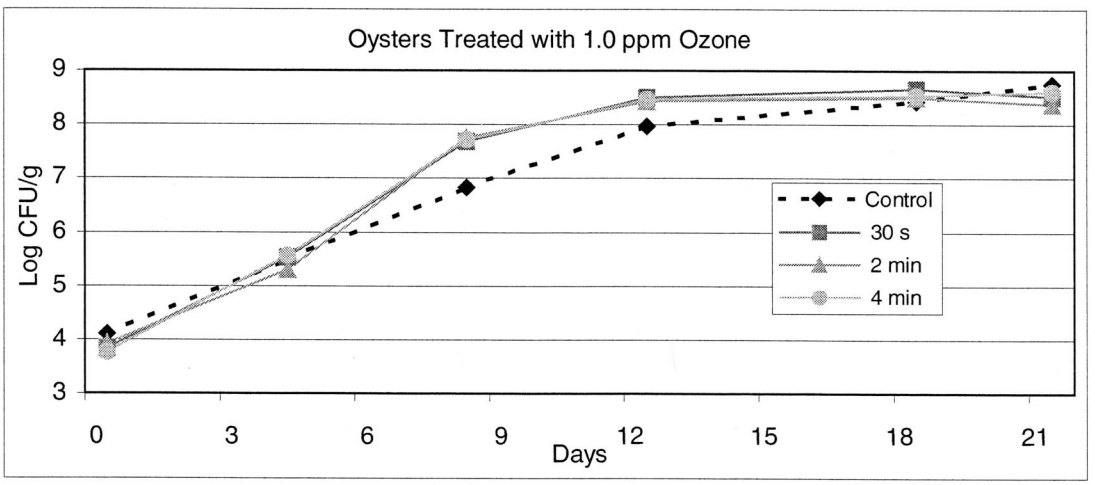
RESULTS

Oysters

The microbial quality of oyster meats washed in 15°C non-ozonated and ozonated water was analyzed by conducting PC and coliform counts. Throughout the 21-day shelf-life study at 5°C, a 4.7-log increase was observed in the control

oyster meats. Treated oyster meats showed no decrease in microbial load compared to the control oyster meat samples (Fig. 3.6A-C). The differences in plate counts between the different treatments and the controls were minor. No increase in shelf-life was observed.

A 3.9-log increase in PC was observed in the control oysters throughout the 21-day shelf-study of the oysters treated at 5°C (Fig. 3.7A-C). Ozonated water (0.5 ppm) was the only treatment to consistently produce a lower microbial load when compared to the control, water washed, and 1.0 ppm O_3 treated oyster meats (Fig. 3.7B). The variability between the PC in the samples washed at 0.5 ppm and 1.0 ppm O_3 could be due to the different initial microbial loads of each oyster. Non-ozonated and ozonated treated oysters showed minor log differences between wash treatments. To illustrate differing initial microbial loads of samples: He and others (2002) found a lower PC from the same genus of oysters, harvested from the same area, using the same microbial and storage methodologies as the present study. They found PC for untreated oysters at day 2 of shelf-life to be 2.5 log CFU/g whereas the present study showed an initial PC to be 4.5 log CFU/g.



A

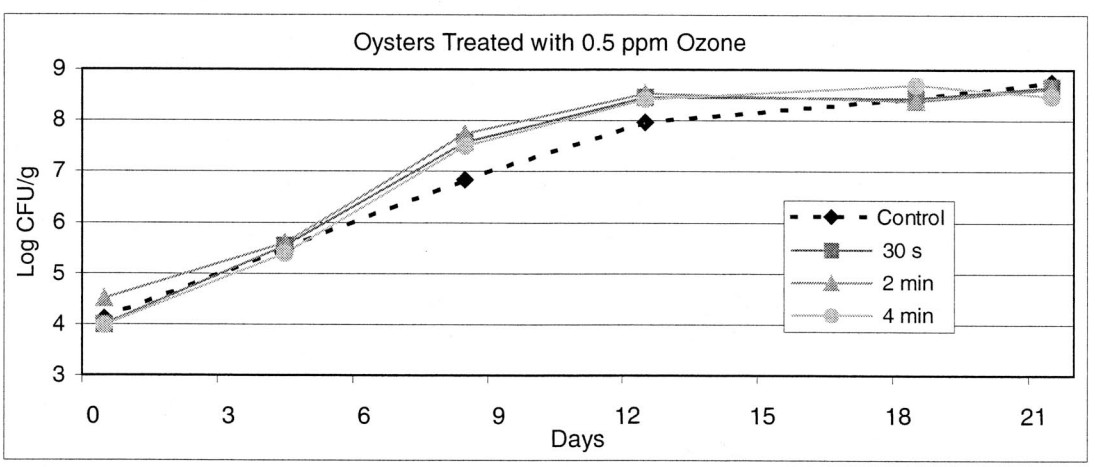


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Figure 3.6. Psychrotrophic plate counts for oysters treated at 15°C.

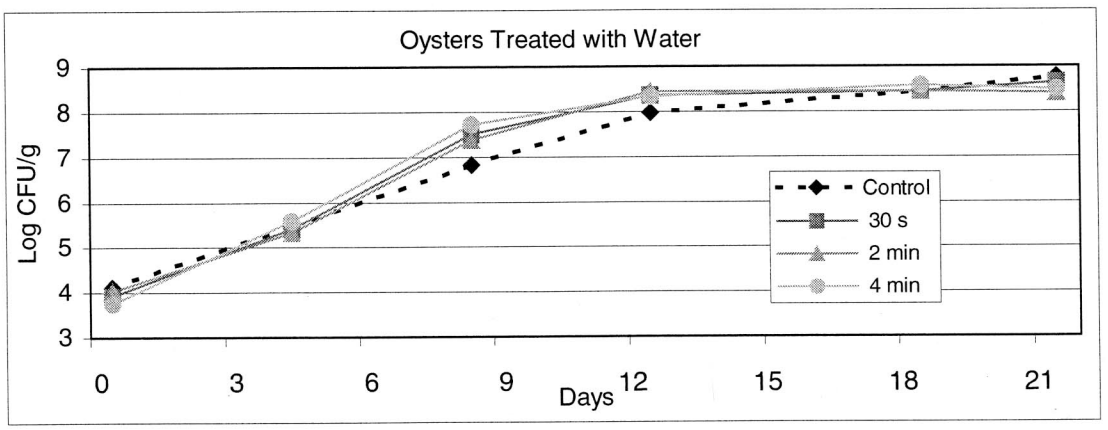

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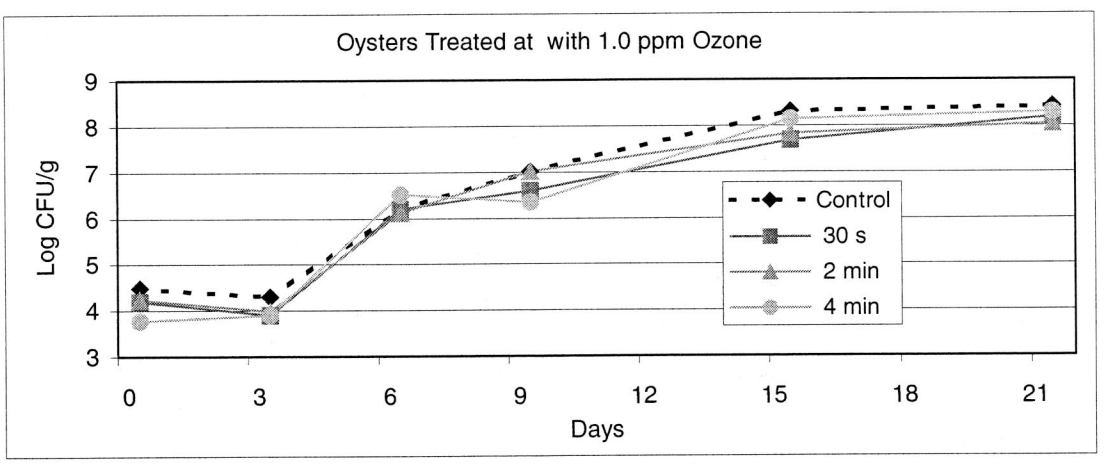
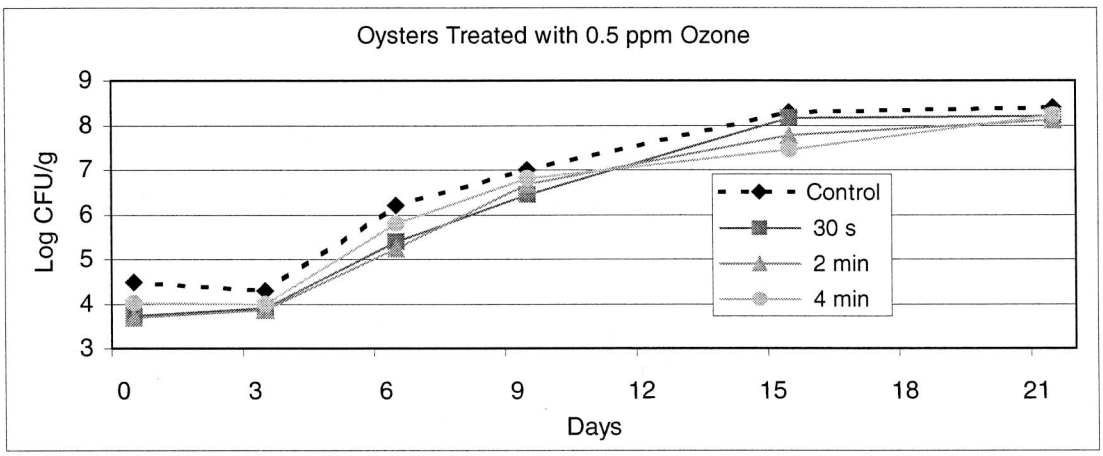
Figure 3.6. Psychrotrophic plate counts for oysters treated at 15°C, Continued.



A

Figure 3.7. Pyschrotrophic plate counts for oysters treated at 5°C.



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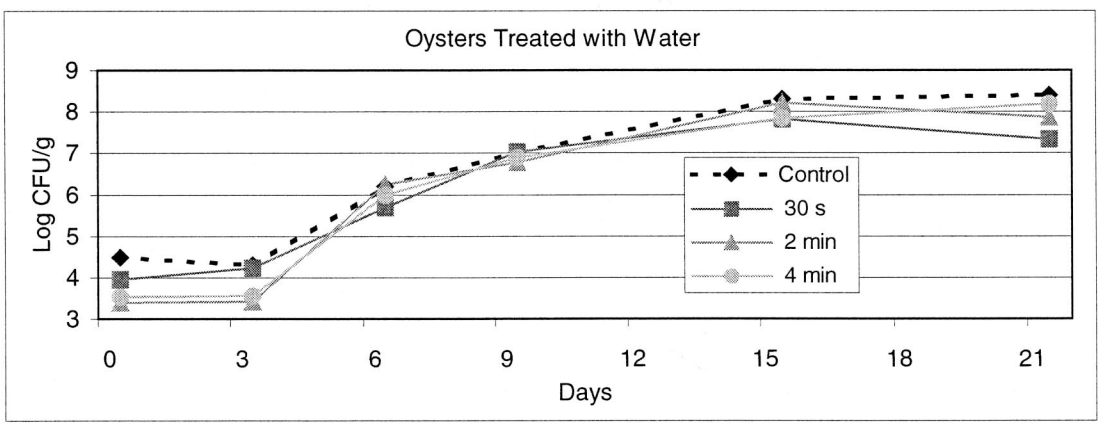


C

Figure 3.7. Pyschrotrophic plate counts for oysters treated at 5°C, Continued.

Throughout the 5°C 21-day analysis, the coliform counts remained low (<10 CFU). The coliform counts in this study were low compared to other oyster studies. Thompson and Vanderzant (1976) found initial coliform counts from 153 oyster samples ranged from 0-2,400 (mean 81.3) per g. Pacific oysters stored at 3°C had an average of 3.2 log CFU/g initially and increased to 6 log CFU/g at day 25 (Hoff and others, 1967b).

The initial percent moisture of the control samples treated at 15°C averaged 77±0.66%, which is consistent with 76±1.3% moisture (Lopez-Caballero and others, 2000). Between day 0 and day 4, the moisture values for all the samples increased sharply and then increased by 0.5-1.5% during the remaining days of shelf- life analyses (Table 3.1). The initial percent moisture of the control group was lower than the wash treatment groups because no water was used in the control group.

Table 3.1. Percent moisture for oysters treated at 15°C.

Days	0	4	8	12	18	21
Control	77.2	87.79	89.11	88.91	90.28	90.32
Water 30 s	80.27	89.4	90.01	89.8	89.84	90.6
2 min	79.33	89.13	88.5	89.51	90.64	91.76
4 min	79.81	88.7	88.98	90.04	90.34	92.46
0.5 ppm ozone 30 s	78.87	89.04	89.48	90.74	89.4	90.21
2 min	80.82	88.18	90.29	90.32	91.01	91.86
4 min	80.66	88.88	90.57	89.66	91.74	91.36
1 ppm ozone 30 s	78.73	89.34	90.28	90.49	90.4	90.95
2 min	79.2	89.4	89.52	90.36	91.33	91.73
4 min	80.68	88.83	90.28	89.28	91.11	90.71

The control samples at 5°C had an average initial moisture content of 74±0.8%. The average percent moisture of all samples increased at day 3 then stabilized, except for the control samples, which continued to increase throughout shelf-life (Table 3.2). The control moisture values from both temperatures treatments in this study slowly increased over time, whereas, a study on the same genus of oysters found that the control moisture values slightly decreased over time (He and others, 2002).

Table 3.2. Percent moisture for oysters treated at 5°C.

Days	0	3	6	9	15	21
Control	74.15	82.79	83.45	84.6	87.53	90
Water 30 s	77.5	83.97	86.51	87.37	86.16	86.91
2 min	77.82	85.45	85.93	86.37	86.03	85.73
4 min	82.4	88.02	85.38	86.71	86.12	87.87
0.5 ppm ozone 30 s	79.76	83.5	84.68	83.46	84.82	85.43
2min	78.68	84.34	83.1	83.13	85.54	85.75
4min	77.83	82.6	82.75	84.32	85.33	87.96
1.0 ppm ozone 30 s	77.39	84.71	83.5	83.5	84.86	85.33
2 min	77.98	84.46	83.54	83.84	87.27	88.07
4 min	78.7	84.49	82.96	83.73	83.09	87.58

The treated samples (ozonated and non-ozonated) had TBA values that fluctuated during the shelf-life study while the control samples remained more constant. These results are consistent with a related study where raw oysters did not show an increase in TBA values during storage at 4°C but fluctuated erratically until bacterial spoilage was evident (Schwartz and Watts, 1956).

The TBA values were slightly higher for oyster meats treated at 5°C than those treated at 15°C, however the values at 5°C were less variable and more consistent. At both temperatures, the control curve was consistent (no sharp increases or decreases in rancidity), as those samples were not subjected to a change in temperature.

The average pH for all samples (control, ozonated and water) treated at 15°C at day 0 was 6.63±0.1 and dropped to an average of 4.13±0.1 by day 21 (Table 3.3). On day 12 there were slight pH increases in the non-ozonated and ozonated treated samples and a large increase in the control oyster pH.

Table 3.3. pH values for oysters treated at 15°C.

Days	0	4	8	12	18	21
Control	6.53	5.51	4.95	5.73	4.33	4.31
Water 30 s	6.64	5.52	4.48	5.00	4.40	4.23
2 min	6.29	5.65	4.63	5.15	4.33	4.09
4 min	6.68	5.86	4.41	5.09	4.22	4.05
0.5 ppm ozone 30 s	6.70	5.57	4.35	4.90	4.31	4.18
2 min	6.72	5.78	4.37	4.86	4.19	4.11
4 min	6.62	5.67	4.78	5.04	4.13	4.04
1.0 ppm ozone 30 s	6.68	5.81	4.38	4.60	4.29	4.12
2 min	6.75	5.90	4.59	4.63	4.29	4.08
4 min	6.73	5.92	4.28	4.72	4.24	4.18

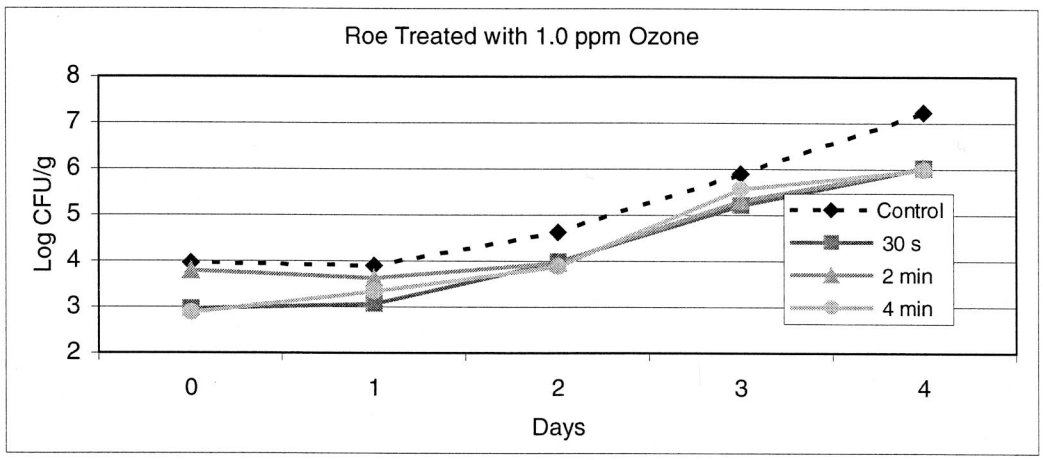
The pH of all the samples decreased an average of 1.67 units from the average initial value of 6.32±0.03 for samples treated at 5°C (Table 3.4). The decline of pH was slower for treatments at 5°C (Table 3.4) than for oysters treated at 15°C (Table 3.3). There is a significant inverse relationship ($P<0.005$) between pH and PC growth of oysters treated at 5°C and 15°C.

Table 3.4. pH values for oysters treated at 5°C.

Days	0	3	6	9	15	21
Control	6.35	6.13	6.06	5.99	5.51	4.36
Water 30 s	6.42	6.18	6.17	6.03	5.78	4.46
2 min	6.33	6.29	6.19	6.06	5.78	5.76
4 min	6.36	6.29	6.17	6.08	5.8	4.56
0.5 ppm ozone 30 s	6.29	6.19	6.07	5.99	5.65	4.69
2 min	6.34	6.19	6.07	6.04	5.76	4.73
4 min	6.3	6.17	6.11	6.02	5.66	4.53
1.0 ppm ozone 30 s	6.26	6.15	6.08	6.01	5.68	4.54
2 min	6.25	6.2	6.04	6.02	5.52	4.48
4 min	6.26	6.21	6.11	6.04	5.73	4.47

Roe

The microbial quality of unprocessed, non-ozonated and ozonated roe was evaluated at 15°C using PC and coliform counts. All treatments resulted in lower microbial counts when compared to the control samples (Fig. 3.8A-C) with the exception of one sample from day 0 treated with 0.5 ppm O_3 for 30 s. The differences in the PC between the different treatments and the control were minor (<1 log).


Roe Treated with 1.0 ppm Ozone
Log CFU/g
8
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Days
Control
30 s
2 min
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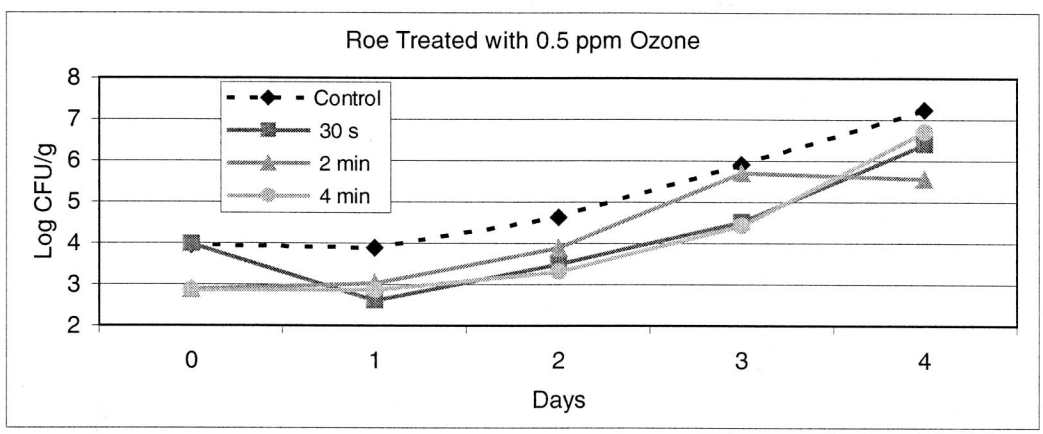
Roe Treated with 0.5 ppm Ozone
Log CFU/g
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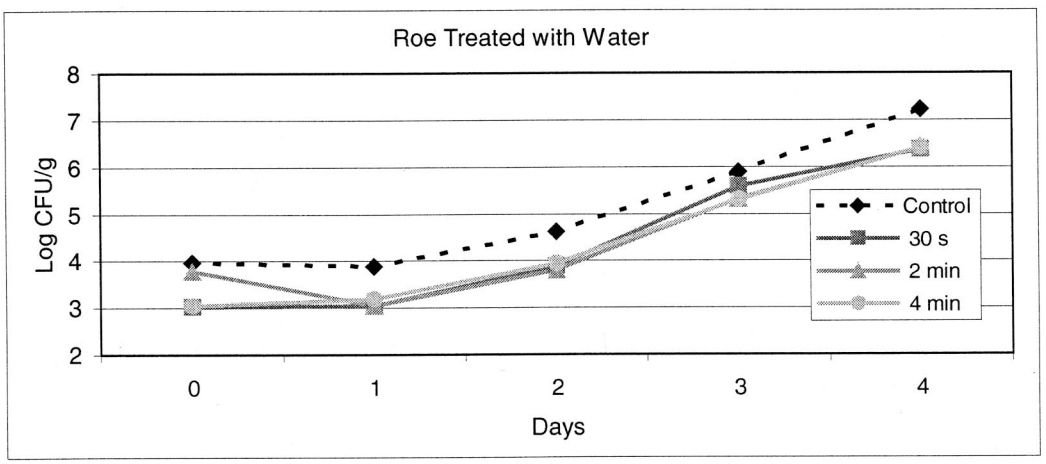
2
0
1
2
3
4
Days
Control
30 s
2 min
4 min

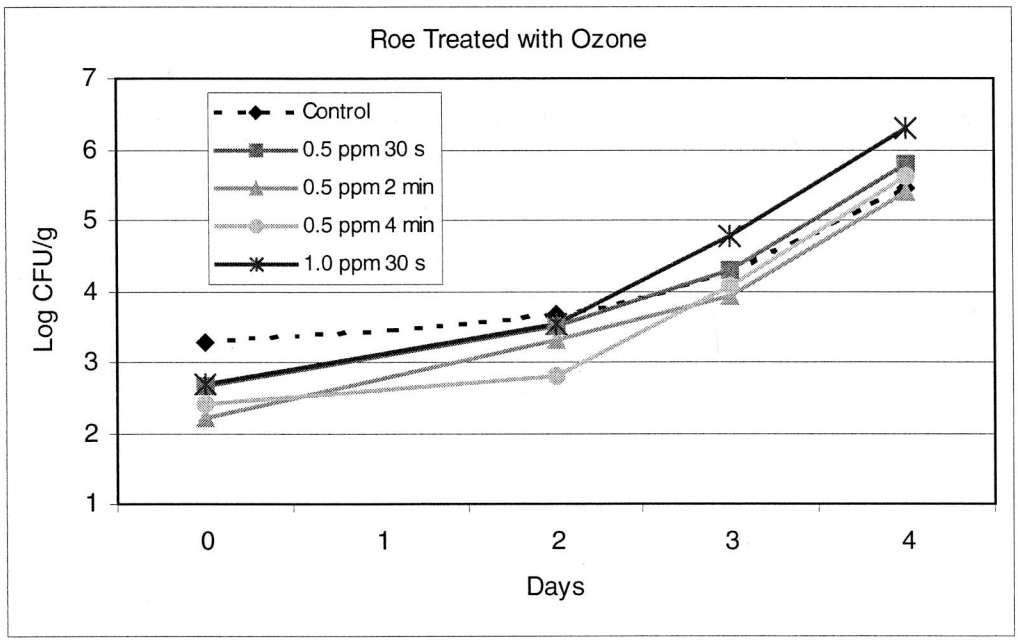
B



C

Figure 3.8. Pyschrotrophic plate counts for salmon roe treated at 15°C.

Decreasing the wash temperature to 5°C did not result in a substantial decrease in the PC of unprocessed roe as compared to the control samples. The temperature drop did result in slowing down the microbial growth rate (Fig. 3.9A, B). By day 2, the 5°C treated samples had microbial counts of 2.8-3.5 log CFU/g whereas the 15°C treated samples had microbial counts of 4.0-3.5 log CFU/g. Initial bacterial counts were reduced by < 1 log by water washing or ozone treatments. The coliform counts from all samples were negligible at <10 CFU/g throughout shelf-life tests.



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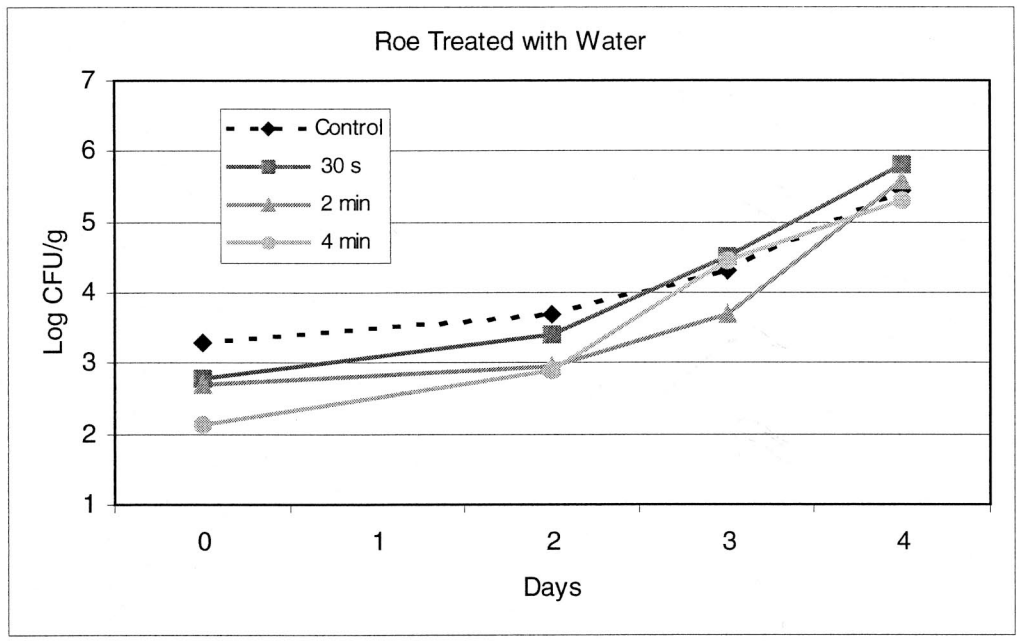

B

Figure 3.9. Pyschrotrophic plate counts for salmon roe treated at 5°C.

The moisture content (Table 3.5) showed minimal change and variability throughout the shelf-life study. The average initial moisture content of all the samples was 60.1±0.9% and the final moisture content was 60.28± 1.1%. The percent moisture of the control group was lower than the treated samples (non-ozonated and ozonated), which could be a result of the control samples not being washed.

Table 3.5. Percent moisture for salmon roe treated at 5°C.

Days	0	2	3	4
Control	58.63	58.15	57.47	58.08
Water 30 s	59.65	59.4	59.59	59.76
2 min	59.93	60.47	60.77	60.7
4 min	60.58	62.52	61.61	61.11
0.5 ppm ozone 30 s	60.4	61.46	60.24	59.71
2 min	59.71	62.8	59.35	60.39
4 min	61.64	60.67	61.58	61.63
1.0 ppm ozone 30 s	60.43	59.9	60.01	60.86

The TBA values from roe treated at 15°C (Table 3.6) were evaluated. Non-ozonated samples were close to or lower than the control, whereas, the ozone treated samples showed more sparatic results. The TBA values increased with an increase in the concentration in ozone. A sharp increase in the control was seen at day 5 except for samples treated with 1.0 ppm ozone.

Table 3.6. TBA values (μg MDA/g roe) for salmon roe treated at 15°C.

Days	0	1	4	5
Control	2.72	0.99	1.46	8.15
Water 30 s	1.83	2.02	0.93	5.16
2 min	2.84	1.29	0.73	4.37
4 min	2.27	1.04	0.95	6.08
0.5 ppm ozone 30 s	1.43	2.98	0.8	2.25
2 min	0.84	2.65	0.8	2.76
4 min	1.59	2.26	0.87	1.96
1.0 ppm ozone 30 s	1.67	9.96	1.1	1.79
2 min	1.62	6.06	1.08	2.65
4 min	0.49	7.65	0.79	1.82

TBA values from samples treated at 5°C (Table 3.7) showed similar trends but were less pronounced in the 15°C studies. Non-ozonated sample values were similar or lower than the control values, whereas, the ozone treated sample values varied, having values both higher and lower than the control sample values with the lower temperature. By day 2, all ozonated sample values were lower than the control, except for the sample treated for 4 min with 0.5 ppm ozone.

Table 3.7. TBA values (μg MDA/g roe) for salmon roe treated at 5°C.

Days	0	2	3	4
Control	4.17	5.14	5.33	2.42
Water 30 s	4.26	2.51	2.17	2.75
2 min	5.22	3.33	0.94	3.02
4 min	4.23	2.26	1.04	7.13
0.5 ppm ozone 30 s	1.54	4.28	2.28	2.57
2 min	2.79	3.3	1.8	2.73
4 min	1.28	6.49	2.32	2.22
1.0 ppm ozone 30 s	9.79	3.39	3.37	2.37

For all roe samples treated at 15°C the average pH for day 0 was 6.36±0.1 and final shelf-life pH was the same (Table 3.8). There were few differences in pH over the 4-day shelf-life study.

Table 3.8. pH values for salmon roe treated at 15°C.

Days	0	1	2	3	4
Control	6.4	6.3	6.3	6.3	6.4
Water 30 s	6.3	6.3	6.3	6.3	6.3
2 min	6.3	6.3	6.2	6.4	6.4
4 min	6.4	6.3	6.3	6.4	6.4
0.5 ppm ozone 30 s	6.5	6.3	6.3	6.4	6.5
2 min	6.3	6.3	6.4	6.4	6.4
4 min	6.3	6.2	6.3	6.3	6.3
1.0 ppm ozne 30 s	6.3	6.3	6.3	6.4	6.3
2 min	6.4	6.2	6.3	6.4	6.3
4 min	6.4	6.3	6.3	6.4	6.4

The average pH of all samples treated at 5°C, at shelf-life day 0, was 6.3±0.1 and a final pH of 6.2±0.0 (Table 3.9), which is slightly lower than the 15°C roe samples. Similar to the higher temperature study, there were few differences in pH over the study period among all treatment samples.

Table 3.9. pH values for salmon roe treated at 5°C.

Days	0	2	3	4
Control	6.3	6.2	6.2	6.2
Water 30 s	6.2	6.2	6.3	6.2
2 min	6.2	6.2	6.3	6.2
4 min	6.2	6.3	6.3	6.2
0.5 ppm ozne 30 s	6.2	6.2	6.2	6.2
2 min	6.3	6.3	6.2	6.2
4 min	6.4	6.2	6.3	6.2
1.0 ppm ozone 30 s	6.3	6.2	6.2	6.2

Fillets

The microbial quality of thawed salmon fillets treated with non-ozonated and ozonated water at 8-9°C was evaluated using PC and coliform counts. Ozonated (1.3 ppm) and non-ozonated treatments were applied for 2 min. Ozone

did not slow bacterial growth on the fillets (Fig 3.10). The coliform counts were <10 CFU/g until day 10 (Table 3.10). The initial coliform counts from this experiment were in agreement with those reported by Himelbloom and others (1991). There were no differences in microbial counts between treated (non-ozonated and ozonated) and control samples.

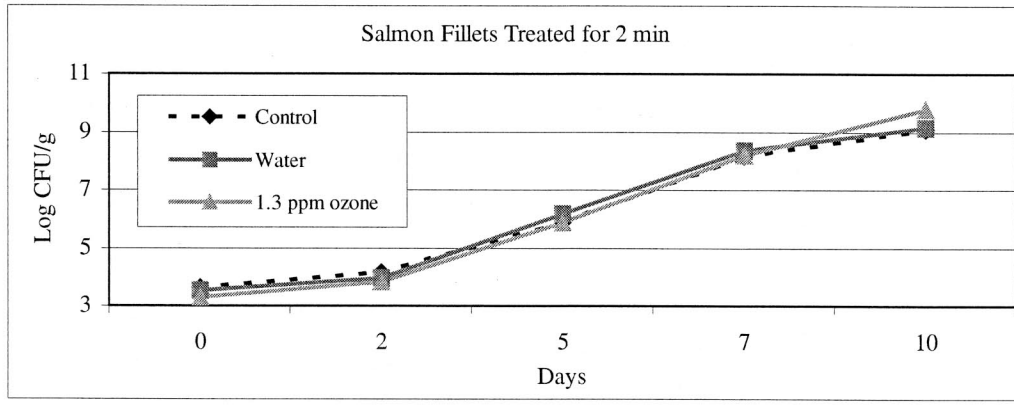

Figure 3.10. Pyschrotrophic plate counts for salmon fillets.

Table 3.10. Coliform counts (CFU/g) for salmon fillets.

Days	0	2	5	7	10
Control	<10	<10	<10	<10	5.39E+02
WW	<10	<10	<10	<10	1.66E+03
1.3 ppm Ozone	<10	<10	<10	<10	6.00E+02

The moisture content of the fillet samples varied between 75-77% throughout the shelf-life analysis (Table 3.11). The unwashed control samples maintained a lower percent moisture.

Table 3.11. Percent moisture for salmon fillets treated for 2 min.

Days	0	2	5	7	10
Control	75.84	76.21	76.05	75.86	76.54
Water	77.67	77.7	76.76	78.19	76.96
1.3 ppm Ozone	76.59	76.37	77.2	77.9	77.12

There were only minor differences in pH among the samples (Table 3.12). These results were similar to those reported for fresh scad treated with ozone (Silva and others, 1998). Both studies showed an initial neutral pH, followed by increased pH levels at the end of shelf-life. However, a study on ozone and Atlantic cod (*Gadus morhua*) reported an increase in pH of 1.5 units from cod washed with 8.0 ppm ozone while the non-ozonated samples only showed a pH increase of 0.6 over a storage time of 20 days (Ravesi and others, 1987).

Table 3.12. pH values for salmon fillets treated for 2 min.

Days	0	2	5	7	10
Control	6.4	6.3	6.4	6.4	6.5
Water	6.4	6.4	6.4	6.5	6.5
1.3 ppm Ozone	6.5	6.4	6.3	6.5	6.6

DISCUSSION

Seafood spoilage is a constant concern in the food processing industry. Effective technology to curb spoilage is needed so that quality products can be provided on a consistent basis. Seafood spoils differently than other meats due to the physiological changes in the muscle composition. This study evaluated ozone technology as a decontamination process for oysters, roe for ikura processing and H&G salmon fillets. Water temperature, microbial growth, shelf-life, pH, TBA and moisture were analyzed as direct indicators of ozone efficacy.

Oysters

The relationships of microbial growth, as measured using PC, and temperature in oysters treated with non-ozonated and ozonated water washes

compares favorably with reports on the effects of ozone on microbial flora and pathogens. Herbold and others (1989) reported that ozone was less effective in water for hepatitis A virus and *E. coli* reduction when the treatment temperature was increased from 10°C to 20°C. Temperature and ozone solubility are inversely related; the lower the temperature of the aqueous ozone, the higher the ozone solubility (Bablon and others, 1991). Temperature variablility was seen as more important than the ozone concentration for reduction in the microbial load of the oyster meats.

All oysters treated at both 15°C and 5°C showed an increase in bacterial growth by day 4. A decrease in the temperature of the treatments at 5°C resulted in a decrease in the bacterial growth rate. All treated (ozonated and non-ozonated) oyster samples in the 5°C experiment, had lower PC than the control groups. Between days 0 and 3, the 5°C samples showed a slower increase in PC compared with the counts from the samples at 15°C. A similar sharp increase in bacteria at day 4 was reported for oysters in a refrigerated shelf-life study (Hoff and others, 1967b). With the exception of one sample, none of the treated samples at 5°C surpassed the microbial growth of the control samples.

The percent moisture for oysters showed an initial sharp increase and then increased gradually during shelf-life studies at both 15°C and 5°C except for an initial sharp rise. The oysters were submerged and stored in water and the osmotic pressure outside the oysters was higher than inside the oysters (Shui, 1999). The

increase in moisture could have been a result from the physical breakdown or simple osmotic balancing of oyster tissue and the increasing amount of water uptake in the oyster meats over time. The initial percent moisture of the control samples was lower then the rest of the samples and could be due to the lack of treatment by non-ozonated or ozonated water washes. The variation in percent moisture between the control and the treated oysters decreased over time, as all samples were stored in jars of water. The moisture values for oysters treated at 15°C were higher than oysters treated at 5°C. The increase in temperature could have caused the more rapid oyster tissue breakdown that could account for the increase in percent moisture at 15°C.

The most widely used quantitative method for measuring the extent of oxidative deterioration of lipids in muscle food is the TBA test (Rhee, 1978; Romijn and others, 1991). The TBA method used in the present study was developed for fresh or frozen fishery products (Sinnhuber, 1958). In this and other studies on raw oysters (Schwartz and Watts, 1956) little consistency in TBA values was seen during storage of samples at 4°C. Seafood spoilage due to microbial growth prior to notable amounts of oxidative rancidity has also been reported (Olcott, 1962). Oxidative rancidity by TBA tends to be measured in months (i.e. frozen seafood) and not in days as found in the present studies.

Bacterial growth and pH decline for oyster meats were strongly correlated ($p<0.05$). The pH decreased at a faster rate with an increase in temperature. All of

the samples at 15°C had microbial counts over 10^7 CFU/g and an average pH of 4.6±0.3 by day 8 whereas the 5°C treated samples had microbial counts of $\leq 10^7$ CFU/g and an average pH of 6.0±0.03 at day 9. Oysters that have a pH of 5.0 or below are considered spoiled (Hunter and Linden, 1923; Cook, 1991). He and others (2002) reported an APC of 7.5 log CFU/g and a pH of 5.9 at day 13 in an oyster shelf-life study.

The chemical composition of mollusks and muscle physiology differs from fish and crustacean shellfish. Mollusks have a significant level of carbohydrate as glycogen (Lopez-Caballero and others, 2000). The glycogen in oysters may have promoted the growth of lactic acid bacteria, which could have caused the decrease in pH and an increase in bacterial numbers. Gram-negative *Pseudomonas* and *Vibrio* and gram-positive *Lactobacillus* bacteria have been reported as the predominant microorganisms involved in the spoilage of oysters (Pascual, 1992). Amylolytic lactic acid producing bacteria hydrolyze glycogen in mussels and produce higher amounts of lactic acid when glycogen is used as energy under controlled conditions as compared to starch (Pintado and others, 1999). Because of the significant levels of glycogen, fermentative microbial spoilage can be expected to occur (Jay, 1978). This inverse relationship between PC and pH is a strong indicator for use in oyster spoilage and may have an important application for

future studies. Correlating pH to water temperature should be taken into account if pH is to be used as an indicator of oyster quality.

Correlations between pH and bacterial growth were not found in salmon roe or fish fillets. When Atlantic salmon were studied under starvation conditions, levels of glycogen in the muscle at slaughter decreased and pH increased with increasing starvation time (Einen and Thomassen, 1998). Oyster spoilage is different from roe and fillets. Oysters are more likely to spoil by fermentation due to lactic acid producing bacteria whereas roe and fillets spoil due to psychrotrophic microbial growth. Salmon roe and fillets do not contain significant levels of glycogen and would not be able to support high populations of lactic acid bacteria.

Roe

Treated (non-ozonated and ozonated) samples resulted in minor (<1 log) microbial decreases when compared to control samples. There were slight differences in the microbial reduction from different temperatures. Decreasing the temperature from 15°C to 5°C did not have a substantial effect in decreasing the microbial load, however, it did have a small effect on slowing down the microbial growth rate. Most of the samples treated at 15°C surpassed 10^6 CFU/g at day 4. In the seafood industry, the microbial maximum standard is 10^6 CFU/g, which

indicates that the product is less acceptable for human consumption. Most roe samples treated at 5°C did not reach a microbial load of 10^6 CFU/g by day 4. It has been reported that decreasing the temperature from 20°C to 10°C increases the effectiveness of ozone on the reduction of hepatitis A virus and *E. coli* (Herbold and others, 1989). However, another study found that there was no change in the inactivation rate of *E. coli* due to varying the temperature from 20°C to 5°C (Hunt and Marinas, 1997).

The percent moisture of roe did not increase with the bacterial load over time, differing from the Pacific oyster results. Himelbloom and Crapo, (1998) reported the moisture content of pink salmon roe to be 49.3% ± 0.5, which is lower than the 58-62% moisture found in the present study. The variation in moisture could be an osmotic effect, since the present study evaluated unprocessed roe while the published study investigated processed roe, in which salt had been added. The average roe pH was 0.2-0.4 units higher than roe pH (6.05 ± 0.05) reported by Himelbloom and Crapo (1998) and may be due to sample differences. There was no inverse relationship between pH and bacterial growth for salmon roe. The pH stayed consistent with minimal variation for both temperature experiments. Salmon roe contains very small amounts of carbohydrates and large amounts of protein in which the protein can act as a pH buffer.

Fillets

Fish fillets are susceptible to rapid spoilage mainly due to neutral pH and growth of psychrotrophic bacteria. Psychrotrophic bacteria grow well under refrigerated temperatures. There was no increase in the fillet shelf-life due to reduction of bacterial growth with the use of ozone. All samples had a microbial load near 10^6 CFU/g. The International Commission on Microbiological Specification for Foods (ICMSF) microbial limit for fresh fish is $5x10^5$ CFU per g or cm^2 (ICMSF, 1986).

In the Licciardello and D'Entremont (1987) study, an initial lag time was observed followed by a sharp increase in microbial growth. Initial microbial lag phase depends on temperature (Sikorski, 1990). A lag period of 1-2 days was reported for fish stored at 0°C (Shewan and Hobbs, 1967). Silva and others (1998) recorded a 2-day lag period from the Pseudomonadaceae and obligate psychrotroph growth rates, in a study on fresh scad. Following the 2-day, lag period the Pseudomonadaceae growth rate increased 2 logs every 2 days, which is similar to the microbial trend seen in the present study.

The pH values of all salmon fillets (treated and untreated) were similar to the reported pH of pink salmon (Crapo and Himelbloom, 1999). Treating the fillets with non-ozonated or ozonated water did not produce significant changes in the pH. Some possible reasons are the low amount of glycogen and high amounts of protein

in which protein can act as a pH buffer in salmon and other types of fish. Therefore, pH would not be an effective indicator for spoilage in most species of fish.

Ozone did not have a great effect in reducing bacterial counts with oyster, salmon roe and fillets. In the present study, there was a large amount of organic matter in the wash water after the each treatment. When microorganisms are suspended in an ozone demand-free solution, ozone can interact directly with the bacteria. Low concentrations of ozone were ineffective in lowering the microbial load with *Escherichia coli, Bacillus cereus* and *B. megaterium* when organic matter was present (Broadwater and others, 1973). In ozonated water, death rates among *S. aureus* compared with *L. monocytogenes* and *E. coli* compared with S. *typhimurium,* were not affected by the addition of 20 ppm of soluble starch but were significantly reduced by the addition of 20 ppm of bovine serum albumin (Restaino and others, 1995). Different kinds of organic material affect microbial death rates in ozonated water. The high amount of organic particles in the wash water could account for the insignificant differences between the water and ozonated treatments.

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CHAPTER 4: MICROBIAL CHANGES IN OZONE TREATED SALMON FILLETS

ABSTRACT

Pink salmon (*Oncorhynchus gorbuscha*) fillets were treated with 1.3 ppm aqueous ozone and water at 8-9°C for 2 min and stored up to ten days at 4°C. Bacteria isolated from treated (ozone and water) and control groups at 0, 5 and 10 days of storage were identified using the API20 NE system. The microbial change in the fillet flora did not differ between treatment groups. Gram-positive bacteria were predominant in all groups at day 0. *Pseudomonas fluorescens* and *P. putida* were the predominant bacterial species found from all groups at days 5 and 10.

INTRODUCTION

Bacteria fall into two general groups based on cell wall structure corresponding to the thickness of peptidoglycan. The Gram stain test (Murray and others, 1998a) distinguishes the cell wall type and separates these groups of bacteria. When crystal violet dye is applied to heat fixed cells and remains after the decolorization process, turning the cell purple, the bacteria are classified as Gram-positive. If the stain does not remain after the decolorization process and requires counterstaining with safranin, the cell turns red and is classified as Gram-negative. Most bacterial identification tests are based on the Gram stain technique.

Microbial sensitivity to aqueous ozone varies with bacterial species, cellular envelope constituents, and chemical and various compounds in the solution. Peptidoglycan is made up of linear polysaccharide chains of repeating disaccharides (amino derivatives of simple sugars) of N-acetylglucosamine and N-acetylmuramic acid (Mathews and van Holde, 1995). N-acetylglucosamine was resistant to aqueous ozone at pH 3 to 7 (Perez and others, 1995). Gram-positive bacteria have greater amounts of N-acetylglucosamine due to the thick peptidoglycan layer in the cell walls which could account for the higher ozone resistance as compared to Gram-negative bacteria (Khadre and others, 2001). Kim and Yousef (2000) observed that the Gram-positive bacteria *Leuconostoc mesenteroides* and *Listeria monocytogenes* were more resistant to aqueous ozone

than the Gram-negative bacteria, *E. coli* O157:H7 and *Pseudomonas fluorescens*. Studies by Restaino and others (1995) and Moore and others (2000) on other bacterial species confirmed these findings.

Psychrotrophic bacteria, predominantly Gram-negative, are the largest microbial contributor to seafood spoilage (Mayer and Ward, 1991). The majority of bacteria found on surfaces of coldwater fish are psychrotrophic (Hobbs, 1983; Sikorski, 1990). The skin microflora of coldwater fish are predominantly Gram-negative bacteria, for example: *Psychrobacter, Acinetobacter, Alteromonas, Pseudomonas, Flavobacterium* and *Vibrio* spp. (Hobbs, 1987). Bacterial spoilage occurs primarily on surface tissue (Sikorski, 1990).

The objective of this study was to determine if ozonated water applied to salmon fillets would reduce bacteria selectively. This treatment was hypothesized to decrease the number of ozone-sensitive, Gram-negative bacteria that spoil fish.

MATERIALS AND METHODS

Treatment groups for pink salmon (*Oncorhynchus gorbuscha*) fillets (n = 2) were: unwashed (control), water washed, and ozone treated (1.3 ppm). Treatments

were applied to one fillet for 2 min increments. Applications of ozone were done similar to methods described in Chapter 3. Experiments were conducted in duplicate.

Aliquots (25 g) of fillets from all three sample groups were taken from the outer 1 cm of muscle. The aliquots were aseptically weighed into sterile stomacher bags (Whirl-pak, Nasco, Fort Atkinson, WI) containing 225 ml of 0.1% sterile peptone water (Difco Laboratories, Detroit, MI) and homogenized at 8 strokes/s for 1 min in a masticator (IUL Instruments, Barcelona, Spain). Subsequent serial 1:10 dilutions of homogenates were made using 0.1% peptone. The dilutions were mixed in a Mini Vortexer (MV 1, VWR Scientific Products, IKA Works, Wilmington, NC) in 25 ml test tubes for 30 s and 1 ml samples were aseptically transferred to prepoured Total Plate Count (TPC; Difco Laboratories, Detroit, MI) agar plates.

The TPC agar was used in the spread plate method (Johnson and Case, 1995) for preliminary colony growth and streaking for colony isolation. TPC petri dishes were incubated at 25°C for a minimum of 48 h or until sufficient microbial growth developed. Fifteen randomly picked colonies were isolated from a plate corresponding to each sample. Identification of non-enteric, oxidase-negative, Gram-negative rods were carried out using the API20 NE system (bioMérieux Vitek, Hazelwood, MO). Other studies have used the system for microbial identification (Lee and Deinginger, 2000) and have reported it to be more reliable

than other commercial test kits (Rice and others, 1993). Colonies 18-24 h old were used for Gram staining and the oxidase test. Non-staining by the KOH method for determining Gram reactions was used on 24 h old colonies (Buck, 1982; Powers, 1995).

RESULTS AND DISCUSSION

Identification of microbial flora on salmon fillets was carried out on 0, 5 and 10 days of storage at 4°C (Table 4.1). Fillets treated with aqueous ozone did not result in a shift in the microbial flora from Gram-negative to Gram-positive bacteria. The ozone treatment did not preferentially kill the theoretically more sensitive Gram-negative bacteria (Table 4.1). The results showed that the majority of bacteria identified were Gram-negative on days 5 and 10.

On day 0, more than 50% of the bacteria isolated were Gram-positive bacteria. Many types of Gram-positive bacteria do not grow well at 5°C or at all (Hobbs, 1983). Most seafood shelf-life studies are stored at 5°C and the flora changes to predominantly Gram-negative bacteria (Shewan and others, 1960; Hobbs and Hodgkiss, 1982). During storage the fillets undergo microbial changes and *Pseudomonas* spp. becomes the dominant bacterial genus (Liston, 1980). The

Table 4.1. Microbial composition of salmon fillets after ozone treatment.

Treatment	Storage Day	Number of Colonies			Total Bacteria
		Gram-positive	Gram-negative	Unknown	Log CFU/g
Ozone	0	8	7	0	3.3
	5	1	14	0	5.9
	10	0	15	0	9.8
Water	0	7	6	2	3.5
	5	6	8	0	6.1
	10	0	15	0	9.1
Control	0	7	7	1	3.6
	5	3	12	0	5.8
	10	0	10	5	9.1

microbial flora shifts are illustrated in Tables 4.1 and 4.2. In a study on the shelf-life of cod stored on ice, over 80% of the microbial flora was comprised of *Pseudomonas* and *Alteromonas* by day 10 (Shewan and others, 1960). *Pseudomonas fluorescens* and *P. putida* were the predominant bacterial species found from all three samples at days 5 and 10. Other Gram-negative species identified were: *Brevundimonas vesicularis*, *Burkholderia pseudomallei*, *B. cepacia*, *Chryseobacterium meningosepticum*, *C. luteola*, *Pasteurella* sp., *Sphingomonas paucimobilis*, *S. spiritivorum* and *Moraxella* sp. The common genera included in the Pseudomonaceae family are: *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, *Acinetobacter*, *Moraxella*, and *Commamonas* (Madigan and others, 1997; Murray and others, 1998b).

Table 4.2. Gram-negative bacteria isolated from salmon fillets after ozone treatment and storage at 4°C.

Treatment	Storage Day	Number of Colonies					
		Brevundimonas	*Burkholderia*	*Chryseobacterium*	*Pseudomonas*	*Sphingomonas*	Other G-
Ozone	0	2	0	3	1	1	0
	5	0	0	0	12	1	1
	10	0	1	1	8	0	5
Water	0	0	0	1	1	2	2
	5	0	0	0	6	0	2
	10	0	5	0	10	0	0
Control	0	2	0	0	1	2	2
	5	0	0	0	10	1	1
	10	0	2	2	6	0	0

In the present study, ozone did not preferentially kill Gram-negative bacteria. Ample residual ozone in the treatment water indicated that the initial concentration was adequate. The ozone demand was very high due to a considerable amount of organic load in the water. It is likely that ozone reacted with the bacteria as well as organic material in the fillet. The 2 min ozone treatment time could be a factor in the efficacy of the ozone application. This experiment was carried out in parallel with an evaluation of an expected commercial application time. Through visits to different fish processing plants and discussions with industry personnel, the conclusion was any treatment over 2 min in the processing line would not be

sustainable from a commercial standpoint. For future studies, an investigation of areas where the ozone contact time could be increased would be beneficial in attempting to decrease the microbial load with ozone.

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CHAPTER 5: CONCLUSIONS

Treating oysters, salmon roe and salmon fillets with aqueous ozone did not result in an increase in shelf-life in the pilot plant studies. In general, there was less than one-log reduction in total plate counts after ozone treatment. Applying ozone in a commercial roe processing facility did result in decreasing the microbial load of the pre-processed ozonated samples. The ozone contact times of the pilot plant and commercial studies were similar. Decreasing the temperature from 15°C to 5°C in the oyster and roe studies did result in retarding the bacterial growth rate in oysters. There were no differences in the microbial flora from non-ozonated and ozonated salmon fillets. The high amount of organic particles in the wash water could account for the insignificant differences between the water and ozonated treatments. The ozone contact times might not have been long enough to produce a consistant visible microbial decrease in the seafood. However, any treatment over 2-4 min in the processing line would not be sustainable from a commercial standpoint. For future studies, an investigation of the use of ozone at different processing stages, e.g. raw material holding stage, where the ozone contact time could be increased may prove beneficial in attempting to decrease the microbial load and increase shelf-life.

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APPENDIX

INDUSTRIAL APPLICATION OF OZONE TO PROCESS SALMON ROE

INTRODUCTION

Ikura is a salt brined product that is sold as individual eggs. The Alaskan roe season generally runs from June-October with peak months of production in July – September. A commercial application of aqueous ozone on ikura was conducted for one season (2000) of salmon roe production. Roe samples were processed and ozonated at Excursion Inlet Packing (XIP), AK. The processing facility currently uses regular water chlorination as a means of a decontamination rinse step. The company investigated alternative technologies that would provide a more efficient method of product decontamination. Commercial processing plant designs are different, therefore the implementation of ozone into the processing water requires flexibility in most processing facilities. The purpose of this study was to compare the effects of ozonated water washed roe to non-ozonated water washed roe in a commercial opporation.

MATERIALS AND METHODS

Ozone Treatment System

XIP protocol for roe in this study included a flow-through fresh water wash system. The ozone generator was set up near the inlet pipe used to transfer water and roe to the processing building. Aqueous ozone was injected into the transfer water near the beginning of the transfer pipe (Fig. A.1).

The transfer pipe was a semi-closed treatment system with one open end approximately 30 m from the injection point. The roe was transported by pipe between buildings via pressurized water for lift and gravity, as the roe had to flow up a short incline. Aqueous ozone was generated and injected into the transfer water to treat the roe (Fig. A.1). The delivery time for the roe after the point of ozone injection was 2-3 min. This system resulted in negligible residual aqueous ozone levels (<0.1 ppm) when the treated roe and water were transported into the roe processing building. To ensure that the ozone levels would meet Occupational Safety and Health Administration (OSHA) regulations, a fan and dewatering box were installed. Prior to the roe processing building, the roe passed through a dewatering box with slats in the bottom allowing the ozonated water to drain and any ozone off-gassing was pulled up into an exhaust fan.

Ozone levels were near depletion at the end of the treatment process and produced a residual that met the OSHA regulations of 0.1 ppm of gaseous ozone over an 8 h exposure time (Damez and others, 1991). A percentage of ozone was diminished when it was injected into incoming processing water. The percentage of ozone decreased further when roe was added to the solution. Ozone will react with organic compounds (besides bacteria), such as food if exposed (Kim and others, 1999).

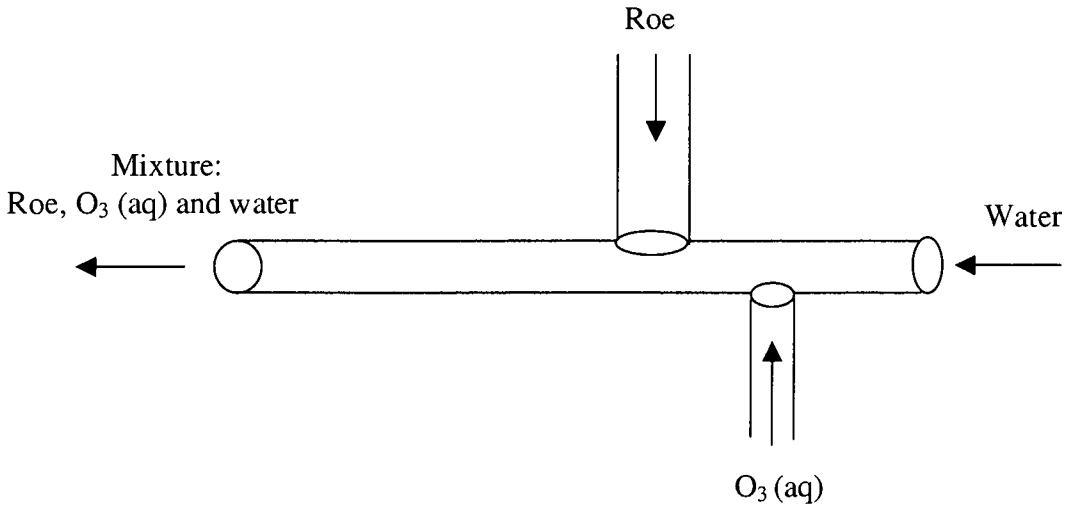

Figure A.1. Roe transfer pipe used as the ozone treatment system.

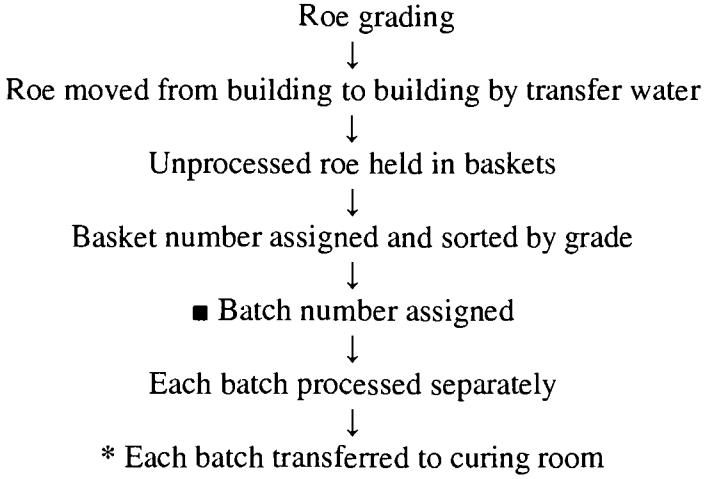

Figure A.2. Ikura processing.
Before processing, ikura was sorted by grade, basket and batch number. Once a batch number had been assigned, the number stayed with the roe through the processing and curing stages.
■ : Pre-processing samples taken
* : Post-processing samples taken

Sampling

Samples were taken from non-ozonated and ozonated pre- and post-processed roe. Pre-processed samples were comprised of both the skein and the eggs. Post-processed samples were comprised of only individual eggs. Each pair of samples was taken from the same grade of roe. Ikura roe was graded before it was transferred to the roe processing building (Fig. A.2). Paired samples were taken from different grades at various times throughout the season. Roe was stored in baskets, allowed to drain and categorized by grade. Each basket holding

approximately 16 kg of roe was labeled with a numbered chip for use in processing reports. Every four baskets of the same roe grade were batched, processed and numbered. All pre-processed samples were comprised of skeins and eggs from all four baskets of a randomly chosen batch. The post-processing samples were taken the following day from the same batch number used for the pre-processing samples. Post-processed samples were taken from the curing room (Fig. A.2).

All samples were collected in zip lock bags (7.62x12.7 cm, Associated Bag Co., Milwaukee, WI) and labeled: pre- or post-processed, non-ozonated or ozonated, batch number, grade, date and sample time. For non-ozonated samples, the ozone generator was turned off allowing only transfer water to bathe the incoming roe. Approximately four samples were taken per day, two pre-processing samples (control and treatment) and two post-processing samples. Occasionally, more samples where taken if a large amount of roe was being processed on any given day.

Microbial Analysis

A total of 45 paired (pre- and post-processed) samples were tested for microbial loads in the in the control and treated roe. The microbial tests carried out on all samples included pyschrotrophic plate count, coliform count and *E. coli* tests.

The 3M Petrifilm (Microbiology Products, St. Paul, MN) was the medium used to perform all of the tests. The coliform (EC) petrifilm was used for all coliform and *E. coli* enumerations and all microbial tests were done in duplicate (McAllister and others, 1988; Matner and others, 1990). Aerobic plate count (AC) petrifilm was incubated at 25°C (to accommodate pyschrotrophic microbial growth) for 48 h, and EC petrifilm was incubated at 35°C for total coliform counts and at 42.5°C for *E. coli* counts.

Ozone

Aqueous ozone was produced from a Zing™ Ozone System (Net Systems, Bainbridge Island, WA). Oxygen was filtered from the surrounding air using an oxygen concentrator then passed through the ozone generator (Fig. 3.3A). Ozone and water were mixed together using a Vortex™ mixing tank (Net Systems, Bainbridge Island, WA).

Ozone concentrations were measured colorimetrically using a DR/850 colorimeter (Hach, Loveland, CO) employing the indigo method (Grunwell and others, 1983). To obtain initial and residual concentrations of aqueous ozone, samples were taken directly from the ozone inlet (vortex mixing tank outlet) and from the ozone outlet (transfer pipe water that entered the roe processing building).

RESULTS

Pre-Processing Roe

Water-washed (chlorinated) samples showed nine samples had microbial loads ≤ 2 log CFU/g and seven samples had microbial loads >3 log CFU/g (Fig. A.3). There were seventeen treated (ozonated) samples with microbial loads ≤ 2 log CFU/g and no samples had microbial loads >3 log CFU/g. No *E. coli* was found in any of the pre- and post-processed samples. Coliform counts were negligible at ≤ 10 CFU/g for all pre- and post-processed samples. The results suggest that ozone decreased the microbial load of roe prior to processing.

Post-Processing Roe

Washing the roe in aqueous ozone did not produce any added benefits in the post-processed roe product. Non-ozonated water washed roe resulted in 26/45 of the post-processed samples with higher microbial loads than the pre-processed

paired samples. Eleven non-ozonated post-processed samples contained ≤ 2 log CFU/g while sixteen contained > 3 log CFU/g (Fig A.3). Aqueous ozone treatment resulted in 24/45 of post-processed samples having higher microbial loads than the pre-processed paired samples. Eighteen post-processed samples (ozonated) contained ≤ 2 log CFU/g while fifteen contained > 3 log CFU/g (Fig A.4). Possible processing contamination may have lead to an increase in the microbial load of the post-processed product.

Ozone

Ozone levels in water during pre-treatment varied between 0.5 ppm and 1.7 ppm. However, the increase in ozone concentration did not result in decreased bacterial loads of roe samples (Fig. A.4).

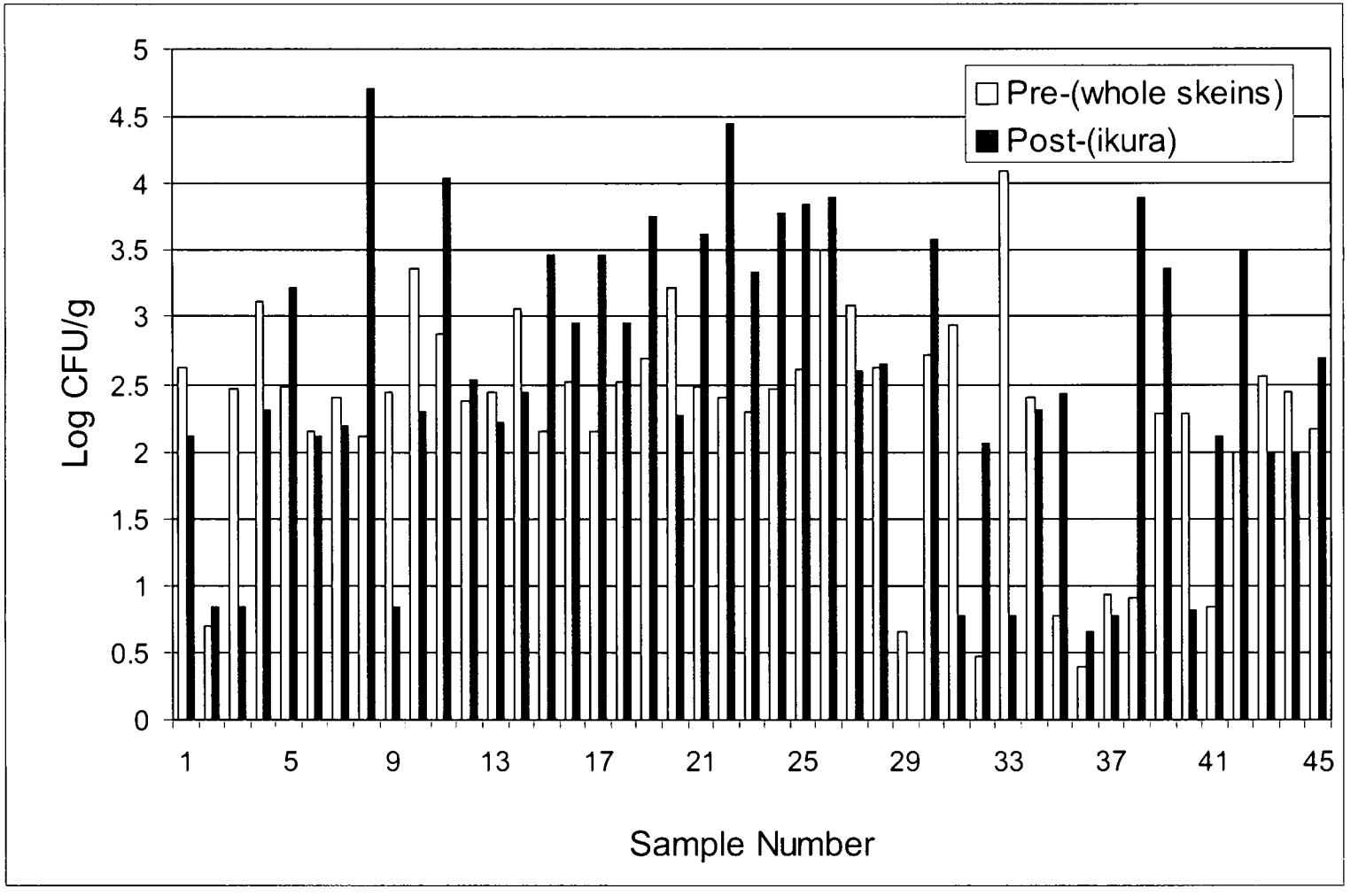

Figure A. 3. Industrial processed salmon roe treated with water.The sample numbers represent a set of paired of pre- and post-samples. Those sets that only have one reading are still paired as any bacterial count that is equal to or less than 1, reads zero in the log scale.

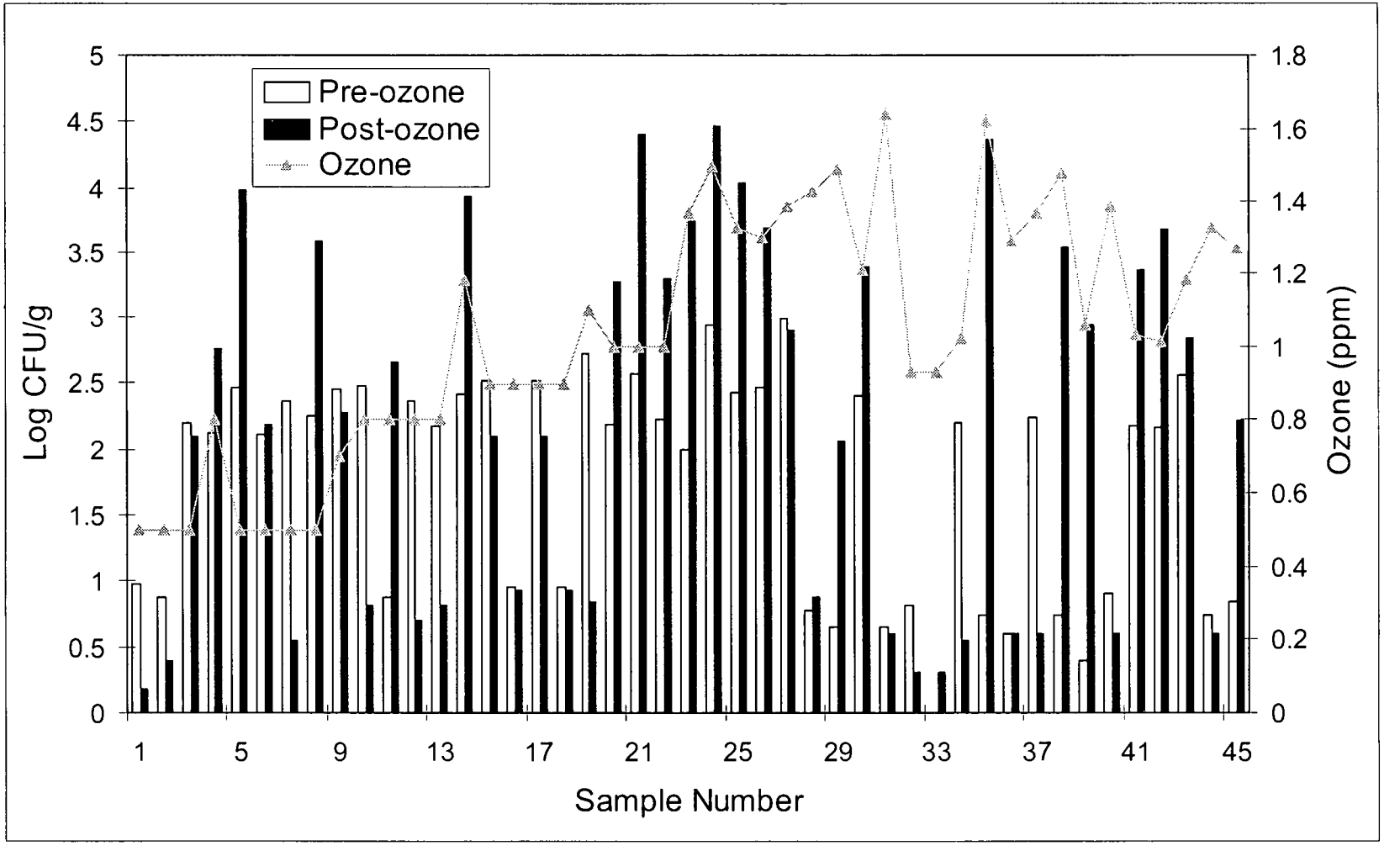

Figure A. 4. The sample numbers represent a set of paired of pre- and post-samples. Those sets that only have one reading are still paired as any bacterial count that is equal to or less than 1 reads zero in the log scale.

DISCUSSION

The results showed that the ozone application used in this study did not decrease the microbial load of processed salmon roe. However, some positive aspects were found for using ozone in an industrial setting. A key factor for decreasing microbial loads could be the consistency of ozone application (continuous supply).

The consistency of ozone treatment (target of > 1.0 ppm) was a significant challenge in a remote, commercial facility due to the lack of uniform water pressure, volume and electrical power. A multi-diesel generator set powered the processing facility in rural Alaska. The water was obtained from a reservoir, which was then treated by filtration and chlorinated before final pumping to the large processing facility. The water pressure in the line varied greatly from the different outlets throughout the facility during varying degrees and types of production. The capability of the ozone generator to produce dissolved ozone was greatly influenced by these variables as at times the minimum requirements for the sytem to generate ozone were not met.

Fluctuations in water pressure and ozone concentration were observed in the ozone generator several times per minute. Additional fluctuations in ozone consistency and concentrations were caused by semi-frequent power outages. The power failures were short and did not cause significant problems in the processing facility. However, when power was cut to the ozone generator there was an

approximate twenty-minute lag period before ozone could be generated at the maximum concentration permissible by incoming water pressure.

Evaluation of the total season's data for Excursion Inlet Packing indicated that continual ozone application (ozone concentration ranged from 0.5 to 1.7 ppm) was more important in decreasing the microbial load of roe than the actual concentration of ozone. These results indicate that ozone helped in decreasing the microbial load of bacteria on pre-processed roe only, although the post-processed roe is the ultimate product reaching consumers. The challenge will be to prevent and to keep the processing area from creating an increase in microbial load in the post-processed product. Ozone treatment is an asset for the product entering into the processing stage, however other technologies must be used to justify the use of ozone in salmon roe processing plants.

Future efforts for using ozone in a commercial roe facility should incorporate new handling strategies, methodologies and technologies throughout the processing steps beyond the pre-processing ozonation step that was tested. Alone, the extent and limitation of this study was not sufficient to justify the use of ozone in salmon roe production.

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