2.3 FISH PROCESSING AND STORAGE

2.3.1 Haraguchi et al. (1969) – Mackerel

Abstract: The preserving effect of ozone was studied with fresh mackerel (*Trachurus trachurus*) and shimaaji (*Caranx mertensi*). In a preliminary experiment, molds, yeasts and aerobic asporogenus bacteria which had been streaked on agar plates were killed after exposure in the atmosphere of ozone (0.6 ppm) for 30-60 minutes. Viable bacterial counts of skin surface of the gutted fish, soaked in 3% NaCl solution containing 0.6 mg/L of ozone for 30 ~ 60 minutes, decreased to 1/100 to 1/1,000 of those of the control fish. The storage life of the fish was lengthened by 1.2 ~ 1.6 times by the ozone treatment once every two days.

Experimental Methods and Results

Action on General Aerobic Microorganisms

Experimental Method: The following 23 strains of tester organisms were used: *Staphylococcus aureus* (209P), *Micrococcus caseolyticus* (KE15), *Serratia marcescens*, *Bacillus subtilis* (PCI), *Bacillus cereus* (IAM-1072), *Escherichia coli* (F-1), *Proteus vulgaris* (YO-1), *Sarcina lutea* (PCI-1001), *Salmonella typhimurium*, *Achromobacter butyri* (NCIB-9049), *Aeromonas harveyi* (NC-2), *Aeromonas salmonicida* (NC-1102), *Pseudomonas aeruginosa* (NCIH-10), *Pseudomonas fluorescens* (NCIB-3756), *Flavobacterium* (SPA-3), *Vibrio parahemolyticus* (3086), *Vibrio alginolyticus* (138-1), *Vibrio anguillarum* (NCMB-6), *Vibrio ichtyodermis* (NC-407), *Saccharomyces cerevisiae* (Sake), *Torula rubra* (Saito), *Aspergillus oryzae*, *Penicillium glaucum*.

After 24 hours incubation (72 hours for true fungi) on slant culture, one platinum-wire loop-full of each organism culture was streaked two lines on a plate containing tryptone agar medium [polypeptone 15 g, wheatone (BBL) 5 g, sodium chloride 5 g, agar 15 g, water 1 liter, pH 7.0], and the halophile culture also was streaked two lines on a plate containing B medium [polypeptone 10 g, Love-lemco beef extract (oxoid) 3 g, yeast extract (Difco) 3 g, sodium chloride 8 g, potassium chloride 10 g, magnesium sulfate 3 g, calcium chloride 1.5 g, agar 12 g, water 1 liter, pH 7.4]. The inoculated plates were placed in a 30 x 30 x 50 cm size airtight container filled with the ozone-containing air allowing plentiful space between the plates, and then ozone-containing air was introduced continuously into the container through an opening located on top of the container. The ozone content in the ozone-containing air was 0.64 mg/L and the treatment temperature was 13EC. The inoculated plates were allowed to contact the ozone-containing air for 15, 30, and 60 minutes in the container. At the end of each treatment period, the plates were taken out of the container and incubated at 30EC for 24 and 48 hours. At the end of the incubation periods, the microbial growth on each plate was determined.

Experimental Results: The results presented in Table 2.3.01 show that 30-60 minutes contact with ozone at the level of 0.6 ppm concentration killed all of the non spore-forming aerobic microorganisms indicate that ozone is effective in killing and controlling a wide range of microorganisms.

Effect of Ozone Treatment on Preservation of Fresh Fish

Experimental Method: For the test, Jack Mackerel (*Trachurus trachurus*) were purchased at the Funabashi fish market, Chiba Prefecture, and shimaaji (*Caranx mertensi*) were captured at the Kachihara fish pond, Chiba Prefecture, and immediately transferred to the laboratory in an icebox. The fish viscera were removed, cleaned and water washed prior to the test. For ozone treatment, the ozone-treatment solution was prepared by passing ozone-containing air through 3% NaCl in water solution kept at 5EC for 30-50 minutes prior to immersion of fish in the solution and the ozone-containing air continuously passed through the solution during the entire period of immersion. The Jack Mackerel were divided into 3 lots of 16 fish each. Lot 1 (control) Jack Mackerel were immersed in 3% NaCl in water solution at 5EC for 60 minutes. Lot 2 Jack Mackerel were immersed in the ozone treatment solution at 5EC for 60 minutes. Lot 3 Jack Mackerel were immersed in the ozone treatment solution at 5EC for 60 minutes on the first day; on subsequent days the identical ozone treatment was repeated at the rate of one treatment per two days.

The Shimaaji were divided into 2 lots of 16 fish each. Lot 1 (control) Shimaaji were immersed in 3% NaCl in water solution at 5EC for 30 minutes. Lot 2 Shimaaji were immersed in the ozone treatment solution at 5EC for 30 minutes on the first day; and then the same ozone treatment as on the first day was repeated at the rate of one treatment per two days. At the end of the immersion in the ozone treatment solution, the treated fish were placed in a sterilized storage container of 30 cm diameter and kept at 2EC. During storage, the fish were removed from the storage container every other day, examined visually, and the viable bacterial count, pH, and volatile basic nitrogen were determined. For determination of the viable bacterial count the plate mix dilution method with B-medium was used and incubated at 30EC for 75 hours.

Experimental Results: The microbiological test results on Jack Mackerel (on skin and in muscle tissues) are presented in Table 2.3.02. The ozone treatment greatly decreased the viable bacterial number on the fish surface and also resulted in retardation of the fish spoilage as determined by organoleptic evaluation (Table 2.3.03). The fish which repeatedly received the ozone treatment every other day did not show any significant increase in volatile basic nitrogen during storage. The organoleptic evaluation also showed that the ozone treatment reduced the raw fishy odor. On the other hand, the repeated ozone treatment increased the dried fish odor. There was no significant change in pH of fish during storage.

Shimaaji received a shorter time (30 minutes) immersion in the ozone treatment solution of higher ozone concentrations. The microbiocidal test results on Shimaaji are presented in Table 2.3.04 and the organoleptic changes in Table 2.3.05. The test results show that the effect of the ozone treatment on Shimaaji was about the same as that on Jack Mackerel. The ozone treatment of Shimaaji delayed the increase in viable bacterial count about 4 days, while organoleptic evaluation of the fish showed the delay in spoilage of the fish by the ozone treatment was a little over a week.

Table 2.3.01. Antimicrobial activity of ozone (Haraguchi et al., 1969)

Organisms - Contact time - min - Incubation time - hr	15		30		60		Control	
	24	48	24	48	24	48	24	48
Staphylococcus aureus (209P)	-	∀	-	∀	-	-	+	+
Micrococcus caseolyticus (KE15)	-	∀	-	∀	-	-	+	+
Serratia marcescens	+	+	-	∀	-	-	+	+
Bacillus subtilis (PCI)	+	+	+	+	+	+	+	+
Bacillus cereus IAM-1072	+	+	+	+	+	+	+	+
Escherichia coli (F-1)	-	+	-	-	-	-	+	+
Proteus vulgaris (YO-1),	-	-	-	-	-	-	+	+
Sarcina lutea (PCI-1001)	-	+	-	∀	-	-	+	+
Salmonella typhimurium	+	+	-	∀	-	-	+	+
Achromobacter butyri (NCIB-9049)	-	∀	-	-	-	-	+	+
Aeromonas harveyi (NC-2)	-	-	-	-	-	-	+	+
Aeromonas salmonicida (NC-1102)	-	-	-	-	-	-	+	+
Pseudomonas aeruginosa (NCIH-10)	-	+	-	∀	-	-	+	+
Pseudomonas fluorescens (NCIB-3756)	-	+	-	∀	-	-	+	+
Flavobacterium (SPA-3)	-	+	-	∀	-	-	+	+
Vibrio parahemolyticus (3086)	-	-	-	-	-	-	+	+
Vibrio alginolyticus (138-1)	+	+	-	-	-	-	+	+
Vibrio anguillarum (NCMB-6)	-	-	-	-	-	-	+	+
Vibrio ichtyodermis (NC-407)	-	-	-	-	-	-	+	+
Saccharomyces cerevisiae (Sake)	-	-	-	-	-	-	∀	+
Torula rubra (Saito)	-	-	-	-	-	-	-	+
Aspergillus oryzae	-	-	-	-	-	-	-	+
Penicillium glaucum	-	-	-	-	-	-	-	∀

Ozone treatment of Shimaaji also produced the dried fish odor near the end of the storage, but there was no significant change in pH of the fish.

Table 2.3.02. Viable Counts on Jack Mackerel Treated With Ozone (data taken from curves) (Haraguchi et al. (1969)

Days of storage	Viable Counts on Skin (x 10^4) per cm^2		
	Control	**Initial O_3 treatment**	**Ozone every 2 days**
0	100	1	2
2	200	10	20
4	9,000	10,000	80
6	20,000	10,000	2,000
8	100,000	10,000	1,000
	Viable Counts in Muscle (x 10^4) per cm^2		
0	0.5	0.5	1
2	5	3	8
4	100	100	5
6	100	200	80
8	1,000	2,000	500

Table 2.3.03. Organoleptic Changes in Jack Mackerel Treated With Ozone (Haraguchi et al. (1969)

Days of storage	Control	Initial O_3 treatment	Initial O_3 treatment
2	Normal	Normal	Normal
4	Slight off-odor Slight softening Eye slightly sunken	Slight softening	Normal
6	Putrid odor Softening of muscle Surface bacterial colonies Skin discolored Eye sunken in orbit	Same as control	Normal
8	Strong putrid odor Softening Abundant surface slime Skin turned white	Same as Control	Off-odor Slight softening Surface bacterial colonies Eye sunken in orbit

Table 2.3.04. Viable Counts on Shimaaji Treated With Ozone (data taken from curves) (Haraguchi et al. (1969)

Days of storage	Viable Counts on Skin (x 10^4) per cm^2	
	Control	**Ozone every 2 days**
0	1	0.02
4	0.01	0.02
8	10	0.02
12	5,000	2,000
16	not measured	800
20	not measured	10,000
	Viable Counts in Muscle (x 10^4) per cm^2	
0	0.0005	0.0005
4	0.01	0.01
8	0.2	0.8
12	1	10
16	not measured	500

Table 2.3.05. Organoleptic Changes in Shimaaji Treated With Ozone (Haraguchi et al. (1969)

Days of storage	Control	Ozone treated every 2 days
4	Normal	Normal
8	Normal	Normal
10	Off-odor; slight softening	Normal
12	Putrid odor; Eye lens turbid; Softening of muscle	Normal
14		Normal
15		Normal
19		Off-odor; Eye slightly sunken in orbit

The results of our test on sterilization of two strains of molds and general aerobic microorganisms show that 30-60 minutes contact with ozone at the same level of concentration killed all of the test microorganisms with the exception of spore-formers. It was recognized that the immersion of fresh fish in a weak salt solution containing dissolved ozone greatly improved the fish preservation. An explanation for this is that a large number of bacteria normally are present on the fish surface and these organisms contribute a great deal to fish spoilage. The viable bacterial population on the fish surface was reduced to 1/100 - 1/1,000 of the original count by the first ozone treatment, but the destruction of bacteria was progressively less as the ozone treatment was repeated; and finally the viable bacterial count on the fish which received

the repeated ozone treatment increased to a level equal to the control lot of fish which received a salt water immersion only.

The organoleptic evaluation of fish showing the longer delay period in fish spoilage through the ozone treatment, compared with the short delay period in the viable bacterial count increase, may be related to a possible decrease of the objectionable odor by oxidation to trimethylamine oxide of trimethylamine produced by bacteria growing on the fish surface. One undesirable effect of the ozone treatment is that the fresh fish gradually lose the odor characteristic of fresh fish and acquire a dried fish odor. This may be due to the oxidation of fish oil by the ozone treatment.

2.3.2 DeWitt et al. (1984) – Shrimp

The objectives of this study were to

1. evaluate the numbers and types of microorganisms on shrimp stored on ozonated ice,

2. relate any changes to standard chemical analyses related to quality, and

3. assess the oxidizing potential of ozone on the development of melanosis.

This study was conducted in two phases. In the first phase, shrimp were purchased on the Gulf Coast and brought to the laboratory in College Station, TX where they were stored on ozonated ice produced in Tyler, TX and delivered to College Station. The second phase was conducted at a pilot plant in Corpus Christi, TX. The shrimp in this phase were purchased in Corpus Christi and the ozonated ice was produced in the lab at Corpus Christi. These shrimp also were prerinsed in ozonated solutions prior to storage on ozonated ice.

College Station Phase: About 100 lbs of bay shrimp (heads on) were transported to College Station where they were deheaded, rinsed, and sorted into five treatment groups. Each treatment group was stored in a separate 48-quart Igloo cooler with a false bottom. The treatments were stored on one of three types of ice: (1) normal ice, (2) ice made from water with a high ozone concentration (ca 2.0 mg/L) and (3) ice made from water with a low ozone concentration (ca 0.5 mg/L). The five treatments in this study were: (1) Control #1 ice storage, (2) High Ozone #2 ice storage, (3) Low Ozone #3 ice storage, (4) High Ozone Bisulfite-bisulfite rinse followed by #2 ice storage, and (5) Low Ozone Bisulfite-bisulfite rinse followed by #3 ice storage.

Shrimp from each group were tested on days 0, 1, 3, 5, 7, 9, 11, 13, 15 and 17. On each test day, ca 400 g of shrimp were removed from each treatment and tested for (1) aerobic plate count, (2) distribution of gram-positive vs gram-negative organisms, (3) pH, (4) black spot, (5) total volatile nitrogen, and (6) ammonia. The thaw drip also was collected from the coolers as needed and recorded. The shrimp were re-iced on days 4 and 11.

Aerobic plate counts (APC) were obtained by combining 50 g of shrimp with 450 mL of peptone dilution water and spread plating serial dilutions. Duplicate samples were conducted for each treatment group. Blending was accomplished using a Stomacher 400 Lab Blender. The distribution of gram-positive vs gram-negative organisms was obtained by gram staining organisms from duplicate plates of ca 30-60 organisms. After initial typing of organisms, the

remainder of the distributions were conducted by sight identification by color and colony appearance.

Corpus Christi Phase: Fresh bay shrimp were obtained in Port Aransas, transported to the laboratory in Corpus Christi and beheaded. Two samples of ca 50 g each were removed to determine initial bacterial levels. The remaining shrimp were divided into the following treatments: (1) control-sea water rinse, shaved ice storage, (2) Fresh Water-ozonated fresh water rinse, ozonated ice storage, (3) Sea Water ozonated sea water rinse, ozonated ice storage and (4) bisulfite-ozonated sea water rinse followed by a five minute soak in 1% bisulfite solution, ozonated ice storage.

Rinsing was accomplished by placing two pounds of shrimp in a stainless steel colander and pouring approximately 5 gallons of the appropriate rinse water over the shrimp. On-board handling was simulated by shaking and swirling the colander. Four pounds of shrimp were used for each treatment. The treatments were placed in ice chests with approximately 8 pounds of ice and placed in refrigerated storage. Fresh ice of the appropriate treatment was added as required.

Shrimp from each treatment were tested on days 0, 2, 4, 6, 8, 11, 13, 15 and 18. On each test day the shrimp were analyzed for aerobic plate count and evaluated for black spot and off-odors. Aerobic plate counts were made by taking three samples of approximately six shrimp each and placing them in whirl pak bags. The shrimp were weighed and placed in Waring blenders with appropriate amounts of 0.1% peptone dilution water and blended for 1 minute. Serial dilutions were plated on standard methods agar and incubated for 48 hours at room temperature. Black spot evaluations were made on the shrimp while they were still in the whirl pak bags. Off-flavor tests were made while the shrimp were in the ice chests.

Results And Discussion

Bacterial Analyses: The results of bacterial analysis conducted in College Station are shown in Figure 2.3.01. The results for day 0 reflect the initial counts after beheading for the five treatments and clearly show the effect of rinsing on the initial bacterial load. There was a 56% reduction in initial counts due to rinsing the shrimp in a 1.25% bisulfite solution. The treatments that were not rinsed (i.e., control, high ozone and low ozone) had initial counts of 8.7×10^4, whereas the bisulfite- treated shrimp had initial counts of 3.8×10^4. The former treatments should have been rinsed in water as a control against the bisulfite rinse. This would have lowered the initial counts for these treatments and might have affected the overall analysis.

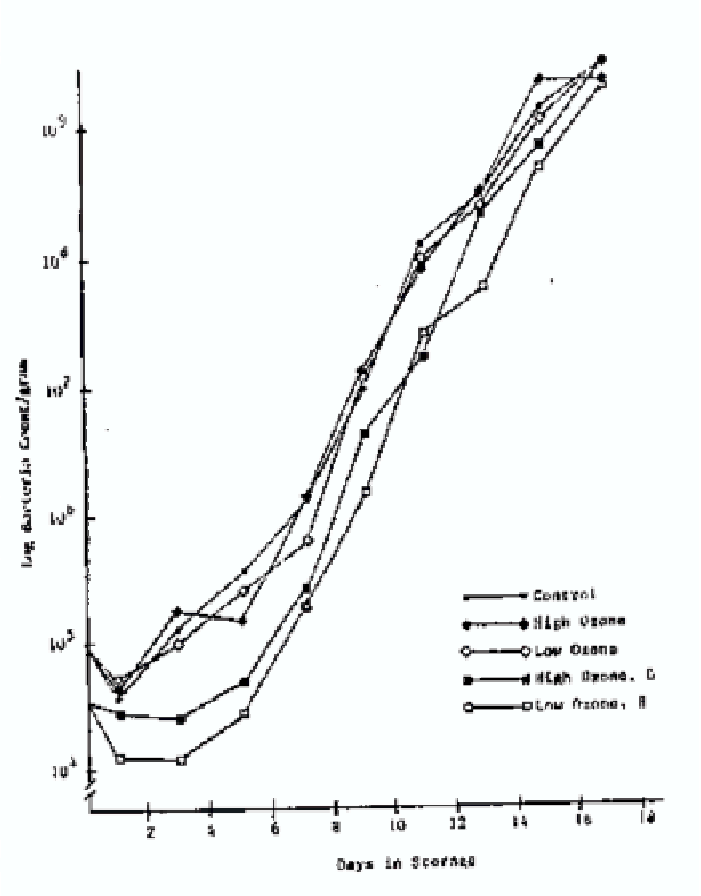

Figure 2.3.01. Log bacteria count vs days in storage for College Station phase of study (DeWitt et al., 1984)

The results of the bacterial analyses conducted in Corpus Christi are shown in Figure 2.3.02. The results for day 0 reflect the initial counts after deheading and rinsing in the appropriate solution. The initial counts for this study, after beheading, were 1.6 x 10^4. Rinsing reduced bacterial numbers to3.7 x 10^3, 2.4 x 10^3, 2.7 x 10^3 and 1.8 x 10^3 for control, fresh water, seawater and bisulfite treatments, respectively. This represents a 77% reduction for the control rinse, an 85% reduction for fresh water, an 83% reduction for sea water and an 89% reduction for the bisulfite rinse. This indicates the effect of ozone in the rinses since the latter three rinses contained ozone. Although the control treatment was significantly higher than the others for day 0, no statistical analysis was conducted to determine whether these reductions were statistically different.

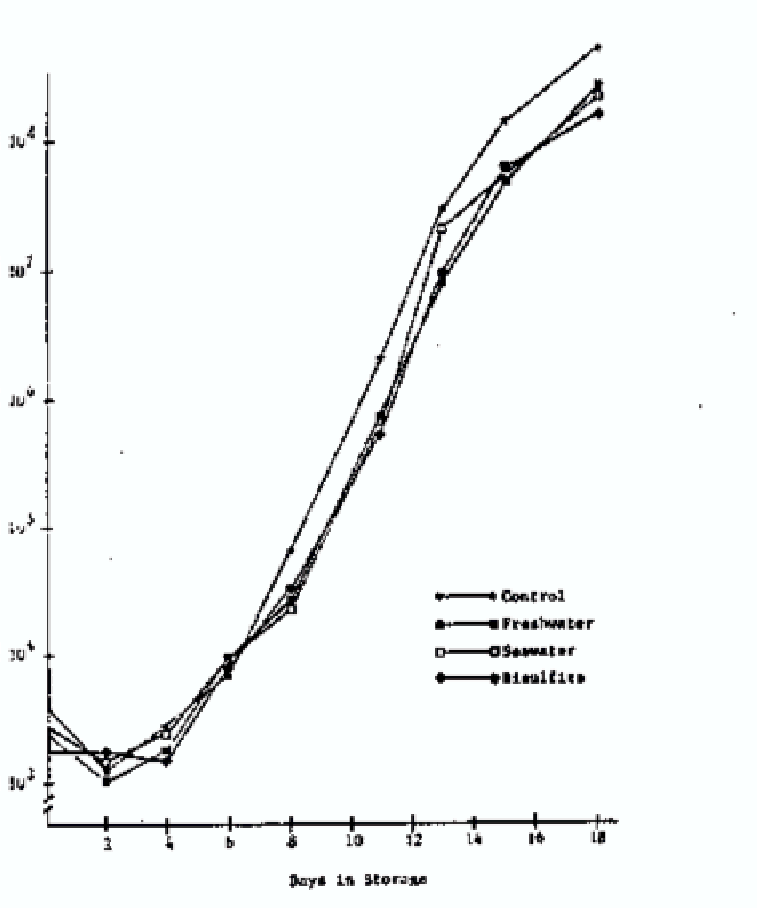

Figure 2.3.02. Log bacteria count vs days in storage for Corpus Christi phase of study (DeWitt et al., 1984)

The difference in initial reductions between the two studies is due to the rinse methods used. In the College Station study, the shrimp were placed in a bucket containing the bisulfite solution and stirred for approximately one minute; whereas in the Corpus Christi study the shrimp were placed in a colander and the rinses were poured over the shrimp. The effect of the rinses in both studies was to extend the lag phase of the bacterial growth for 2-3 days. This apparently had no effect on the shelf life in either study, however it did affect the statistical analysis. The lower counts obtained in the initial part of the studies brought the overall averages down for these treatments and thereby affected the statistical comparisons between the overall means for each treatment.

Both studies were analyzed statistically using analysis of variance and the treatment means were compared. Analysis of variance for both studies showed that there was a statistically significant effect due to the treatment methods. In the College Station study, the two bisulfite treatments had means that were significantly lower than the other three. There was no difference between the two bisulfite treatments and there was no difference between the other three (control, high ozone and low ozone). In the Corpus Christi study, there was no difference between the three treatments using ozonated ice, however the control was significantly higher than all three. This could be attributed to either the ozonated ice or the ozonated rinses.

By studying Figure 2.3.01, it is apparent that there was no effect due to storage on ozonated ice. The only effect in this study was due to the bisulfite rinse, which lowered the initial counts and delayed the lag period of bacterial growth. It can possibly be concluded that the amount of drip water could have affected the results (i.e., delayed lag phase) for the bisulfite treatments since by day 5, 4600 mL of drip water was collected from these treatments compared to only 3600 for the other three. The drip water from the ice has a washing effect on the shrimp and can reduce the bacterial load.

Shrimp from all treatments spoiled at the same rate when the bacteria reached the log or growth phase. At day 13, only the low ozone-bisulfite treatment was different. By day 17, shrimp from all treatments were the same. Therefore it can be concluded that storage on ozonated ice had no effect on the bacterial spoilage of the shrimp used in this study (College Station).

Figure 2.3.02 shows that the bacterial levels in ozonated ice treatments were lower than the control treatment for all days following day 7. This study (Corpus Christi) showed that storage of shrimp on ozonated ice could possibly have an effect on the bacteriological spoilage of shrimp, possibly 1-2 days extension of shelf life. The overall effect of the ozonated rinses could be minimal because the effect did not show up until day 7. The difference between the two studies is that the Corpus Christi study used ice that was ozonated and made on the premises, whereas in the College Station study the ice was made in Tyler (Texas) and shipped to the lab in College Station. The ice used in the former study could have contained residual ozone, whereas the ice used in College Station probably had lost its residual due to transportation and storage.

Distribution of Gram (-) versus Gram (+) Organisms: Table 2.3.06 summarizes the data obtained in this part of the study. The distribution of gram (-) organisms went from approximately 25% to approximately 95% for all treatments. Analysis of variance was not run on this data because the figures show clearly that there was no treatment effect due to the storage of shrimp on ozonated ice. Differences within the observations for certain days can be attributed to the number of organisms isolated for each treatment. The total number of isolates for a particular treatment is solely dependent on the aerobic plate counts for that treatment.

Conclusions:

In the College Station phase of the study, the use of ozonated ice had no effect on the shelf life of shrimp stored on ice. In the Corpus Christi phase of this study, ozonated ice was shown to possibly prolong the shelf life of shrimp stored on ice for 1-2 days. However, this study was unable to distinguish whether the extension of shelf life was due to the use of ozonated rinses or to the use of ozonated ice for storage. The major problem was whether the ice contained residual ozone. In the College Station study, the ice probably did not contain ozone, but in the Corpus Christi phase, there may have been ozone in the ice since the effect or the ozone did not appear until day 7 of the study. The use of ozonated ice had no effect on the incidence of black spot in either study.

Table 2.3.06. Distribution of Gram (+) vs Gram (-) Organisms from College Station Study (DeWitt et al., 1984)

Day	Control		Ozone (H)		Ozone (L)		Ozone (H) Bisulfite		Ozone (L) Bisulfite	
	% G+	%G -	% G+	%G -	% G+	%G -	% G+	%G -	% G+	%G -
0	75.0	25.0	75.0	25.0	75.0	25.0	77.0	23.0	77.0	23.0
1	73.5	26.5	76.0	24.0	77.5	22.5	82.5	17.5	78.0	22.0
3	74.5	25.4	69.5	30.5	75.5	24.5	61.5	38.5	51.0	49.0
5	77.0	23.0	65.0	35.0	68.0	32.0	42.7	57.5	58.5	41.5
7	35.5	64.5	45.5	54.5	49.0	51.0	45.0	55.0	61.0	39.0
9	39.0	61.0	20.0	80.0	30.5	69.5	24.5	75.5	26.5	73.5
11	12.0	88.0	37.0	63.0	10.5	89.5	22.5	77.5	12.0	88.0
13	11.0	89.0	19.5	80.5	9.0	91.0	13.0	87.0	14.0	86.0
15	8.5	91.5	7.5	92.5	8.5	91.5	10.0	90.0	7.0	93.0
17	6.5	93.5	5.5	94.5	6.5	93.5	3.5	96.5	4.5	95.5

2.3.3 Lee and Kramer (1984) – Sockeye Salmon

Abstract: Sockeye salmon (*Oncorhynchus nerka*) obtained from a set net site near Kenai, Alaska were dressed and stored in ice made from chlorinated (2 ppm), low dose (0.86 mg/L oxidant) and high dose (2.32 mg/L oxidant) ozone-treated water. The microbial number increased to 100,000/g in ten days, to 10,000,000/g in 14 days and to 1,000,000,000/g in 21 days. There was little difference in microbial count between the fish washed in chlorinated water and stored in ice made from chlorinated water and those washed with ozonated water and held in ozonated ice. The visual-olfactory scores showed that the salmon retained the acceptable quality for two weeks on ice. The subsequently frozen fish did not develop oxidative rancidity in six months. Very little difference in appearance or quality as evaluated by a taste panel was noted among salmon stored in chlorinated or ozonated ice, except the gills retained fresher appearance much longer in ozonated ice.

Specifically, this study looked at the effectiveness of the ice made from ozone-treated water versus that of ice made of chlorinated water. Secondly it looked at ozone's effect on the development of oxidative rancidity. This investigation was undertaken to compare ozone with another method of treating the water used to wash the fish and to make ice for holding them. To minimize differences within each set of fish due to differences in time of catching or methods of handling, the best quality fish were selected from a batch of fish caught commercially.

Fresh sockeye salmon were obtained at the set net site and chilled promptly in ice. After cleaning and dressing, they were stored in ice made from chlorinated water or water treated with one of two different levels of ozone. The fish were held in ice up to 21 days and samples were examined periodically for microbial load and visual-olfactory attributes. After three and six months of subsequent frozen storage, the samples were examined for flavor and oxidative rancidity.

Materials and Methods

Sample Collection and Preparation: Sockeye salmon (*Oncorhynchus nerka*) were obtained from a beach set net site near Kenai, Alaska. The fish were selected for freshness, lack of physical damage, and uniformity in size. The sample fish were iced immediately at the beach and transported to a nearby processing plant. The fish were no more than one hour out of the water and the early morning ambient temperature at the beach was in the low 50s (EF). The ice to chill the fish during transport to the processing plant was made from untreated artesian well water.

Within an hour of receipt, the salmon was "princess dressed" (head and gills left intact). A portion of the dressed fish then was immersed in chlorinated water at 3 ppm residual level, and in water treated with a low level of (aqueous oxidant residual level of 0.86 mg/L) and a high level (aqueous oxidant residual level of 2.32 mg/L) of ozone. The fish then were placed in layers of ice made from the chlorinated, low-level ozone and high-level ozone water, respectively. Three totes of 48 x 48 x 30 inches containing 2l fish each were kept in a refrigerator overnight at the plant and then were trucked to the laboratory for storage and analysis.

Iced Storage: Totes were stored at 1.7EC ambient temperature with the drain plugs open. Three fish from each tote were removed at 1, 5, 10, 14, 17 and 21 days of storage. The 0 day samples were taken at the Sterling plant, packed in ice and flown to the Palmer laboratory on the same date to be analyzed for microbial contents.

Test Protocol: Fish were removed from the totes (with handlers careful not to touch the lower portion of the fish), placed in a plastic bag, and carried to the laboratory. Upon receipt, a 2 x 2-inch section of belly flap near the anal region of each fish was aseptically excised and placed in a sterile jar. An identical sample was taken from the opposite side of the fish and kept frozen as a contingency sample.

Microbiological Procedure: Each sample of fish flesh was weighed and sterile Butterfield's phosphate buffer diluent was added to obtain one to ten (weight to volume) dilution. The sample then was blended in an Osterizer blender until the meat was finely and homogeneously ground. The blending took between 15 and 20 seconds at high speed. After a serial dilution, appropriate diluents were plated on solidified nutrient agar and spread-plated with a sterile L-shaped glass rod. After incubation at 25EC for 48 hours, the visible colonies were counted and the microbial load calculated.

All colonies on the countable plates then were transferred to a nutrient agar plate on spots that corresponded to the nichrome wire alignment of a replicator. After incubation at 25EC for 24 hours, the regrowths on the agar plate were transferred onto standard method caseinate agar and basal lipolysis agar, This transfer was to determine the proteolytic and lypolitic activities of the microbial isolate.

Three fish were tested individually per treatment at each sampling. The diluent, media, and test procedures were selected to ensure optimum recovery of stressed bacteria. The differentiation scheme was developed based on previous experiences of one of the principal investigators (JSL).

There was insufficient time for a trial run, however, and the test failed to determine the lipolytic activity.

Results and Discussion

Microbial Content of Plant Water: Table 2.3.07 summarizes the microbial counts of untreated artesian well water after chlorination with sodium hypochlorite (household bleach) to 2 ppm residual level in melted ice, and after treatment with two different doses of ozone, at 0.86 mg/L. and 2.32 mg/L. of aqueous oxidant residual levels. The well water contained 194 viable microorganisms per 100 mL. Surprisingly, the ozone treatment did not appear to inactivate microorganisms as effectively as did the 2 ppm chlorine. Part of the microbial load found in ozone-treated water, however, could have been from -an extraneous source.

The lower part of Table 2.3.07 shows an attempt to verify the findings after the fact by analyzing the ice that held the fish samples for two days. The ice had been disturbed during fish sampling and the microbial count reveals the resultant contamination. Nevertheless, the relative abundance of microorganisms in the three ice samples remained the same.

The untreated raw water was negative for both coliform and fecal coliform.

Microbial Count of Washed Salmon: Sockeye salmon was "princesses dressed" and cleaned using chlorinated and ozone-treated waters. A microbial count was made from a 2 x 2-inch section of belly flap taken near the anal region of the fish. After sampling, the same fish was thoroughly cleaned while immersed in the treated water. Fresh samples were again taken from the opposite side of the fish.

The microbial data of this experiment is presented in Table 2.3.08. As can be seen, the second cleaning seems to have further lowered the microbial load, but the fish had already received such care that the effectiveness of additional cleaning was not as apparent.

Microbial Count of Ice-Stored Salmon:

The microbial counts of sockeye salmon stored in treated ice. and sampled periodically for 21 days are presented in Table 2.3.09. These data also are plotted on six-cycle semi-log graph paper in Figure 2.3.03.

Table 2.3.07. Microbial count of ice before and after introduction of fish
(Lee and Kramer, 1984)

Sample	Sample Date	CFU/100 mL [a]
1. Raw water	7/11/83	194
2. Chlorinated water ice (2 ppm)	7/18/83	NG, NG [c]
3. Ozonated water ice (low)	7/18/83	457, — [d]
4. Ozonated water ice (high)	7/18/33	3, 40
5. Chlorinated water ice (0)	7/20/83 [b]	53, (37) [e]. 60, 68
6. Ozonated water ice (low)	7/20/83 [b]	(5, 443), (3, 515), (2, 722), (3, 289)
7. Ozonated water ice (high)	7/20/83 [b]	47, (15), 456, (794)

[a] Colony forming units per 100 mL of water.
[b] Two-day old ice from the tote.
[c] NG = no growth.
[d] One of the duplicate samples contained unfilterable fibrous material.
[e] Number in parenthesis shows contamination from fish visually detectable.

Table 2.3.08. Microbial count of sockeye salmon before and after treated water wash
(Lee and Kramer, 1984)

Sample	Before	After
	CFU [a]/g	
1. Chlorinated water wash	7.9×10^3	1.5×10^3
2. Low level ozonated water wash	1.1×10^3	1.1×10^3
3. High level ozonated water wash	4.6×10^3	2.9×10^3

a Colony forming unit.

Microbial loads of the fish samples obtained from three different ices were remarkably similar. Microbial growth was minimal during the first five days, but it increased exponentially for the next 12 days. After this period the growth started to level off.

The proportion of the proteolytic bacteria found in the three test groups was not distinctly different (Table 2.3.10). This indicated that the microbial population shift in salmon stored in three test ices had followed a similar pattern.

Table 2.3.09. Microbial count of sockeye salmon in treated ice (Lee and Kramer, 1984)

Days	CFU [a]/g		
	Chlorine	Low Ozone	High Ozone
0 [b]	1.5×10^3	1.2×10^3	2.9×10^3
1	8.8×10^2	1.1×10^3	1.5×10^3
5	7.5×10^3	6.6×10^3	4.0×10^3
10	5.5×10^5	2.7×10^5	1.5×10^5
14	2.6×10^7	1.2×10^7	7.6×10^6
17	5.2×10^7	6.3×10^7	1.4×10^8
21	— [c]	6.4×10^8	8.0×10^8

[a] Colony forming unit.
[b] Before icing.
[c] No sample taken due to ice exhaustion.

Conclusions:

1. Ozone was equally effective compared to chlorine.

2. The gills of sockeye salmon appeared fresher whether the fish were held in ice made from ozone-treated water.

3. Properly handled, sockeye salmon could be stored oil ice for at least two weeks.

4. Microbial counts increased in closer parallel to the days the salmon had been stored on ice.

5. Ice made from ozone-treated water did not promote rancidity development in salmon during subsequent frozen storage.

6. Sterility of ice did not appear to be a critical factor.

2.3.4 Chen et al. (1987) – Frozen Fish Products

This article is written in Chinese, but with an English abstract and several figures and tables with legends and numbers in English as well. Pertinent microbiocidal information is presented.

Abstract: This study was conducted to elucidate the sterilization effect of ozone in water of different conditions and of in-plant sterilization of frozen fishery product factories. The results indicated that the sterilization effect of ozone in distilled water or in 3% NaCl solution was higher than that in water containing organic matter. Temperature (5EC and 25EC) did not significantly influence the sterilization effect of ozone in water with same treatment. Within five test microorganisms, *Vibrio cholerae, Escherichia coli*, and *Salmonella typhimurium* exhibited almost the same response to ozone; while *Staphylococcus aureus* was more resistant to ozone. *V. cholerae, E. coli* and *S. typhimurium* were inactivated in water when ozone was flushed for 2 min and the final ozone concentration was 0.7 mg/L. When the concentration in water was 1.4

mg/L after flushing ozone for 8 min, *V. parahaemolyticus* was inactivated. For *S. aureus*, longer time was required for it to be inactivated.

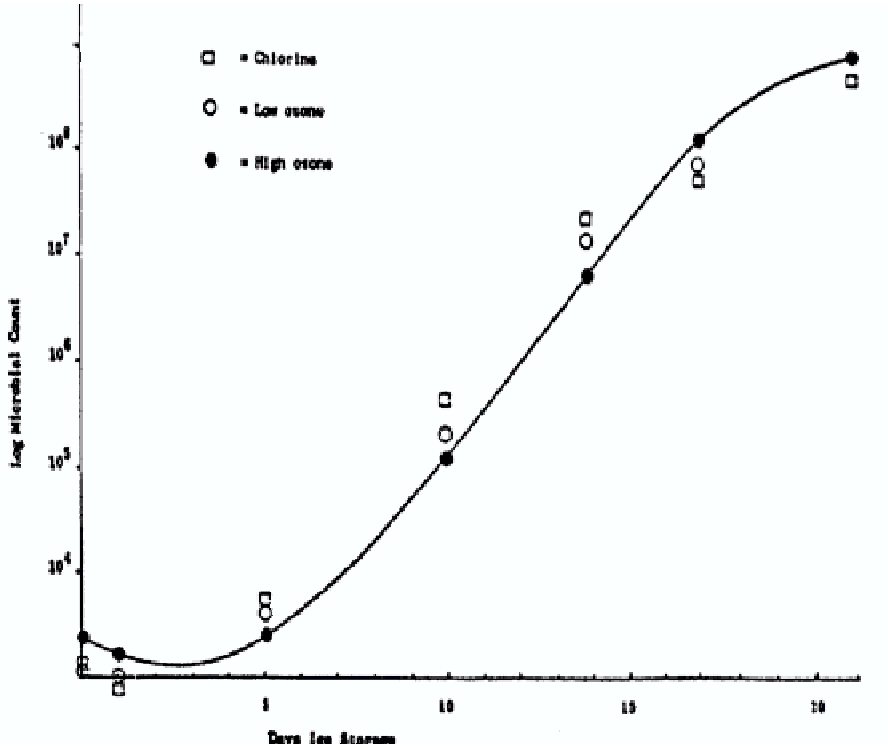

Figure 2.3.03. Microbial counts of sockeye salmon stored in treated ice (data in Table 2.3.09) (Lee and Kramer, 1984).

The sterilization effect of ozone on the bacteria in shrimp meat was not effective. During one-hour-ozone flushing treatment in water containing 3% NaCl at 5.5-6.8EC and at 24EC, *E. coli* levels in shrimp meat were reduced about 98.5% only (see Note 3 below). Therefore, the application of ozone in frozen fishery product industries for raw material sterilization was not effective.

Notes by the FAP Petitioners:

1. Orientals tend to use the word "sterilization" with respect to ozone when they really mean "disinfection". In articles written by Orientals, the reader should not interpret "sterilization" to mean the absence of all microorganisms.

Table 2.3.10. Percent of Proteolytic Bacteria in Sockeye Salmon [a] (Lee and Kramer, 1984)

Sample (days)	Chlorinated Ice	Low Ozone Ice	High Ozone ice
0	84	87	94
1	45	67	70
5	87	79	89
10	22	9	20
14	87	60	78
17	47	57	60
21	— [b]	44	56

[a] Sodium caseinate hydrolysis.

[b] No sample.

2. Table 2.3.11 shows ozone concentrations in waters after "flushing" (passage of ozone-containing gas from the ozone generator through the water samples) at different conditions. Of particular interest are the last two columns. The second-to-last column is captioned "0EC water + 300 ppm milk". Although the meaning of the word "milk" is not clear from the available English text, it is assumed that the material is organic in nature and exerts some demand for ozone. Comparing ozone concentrations in distilled water at 0EC (first column) and at 0E + 300 ppm "milk", the ozone levels in the "milk"-containing water are much lower than those in distilled water.

3. The last column of Table 2.3.11 is captioned "0EC water + 0.3% NaCl" and lists "ozone concentrations" in this water at least double those of distilled water at the same temperature. Such high concentrations in the last column are unexpected, assuming that the method for determining dissolved ozone was specific for ozone. Lacking confirmation for that assumption, one explanation for the unexpectedly high "ozone" concentrations is the possible presence of some bromide ion in the NaCl used. For example, sea water is known to contain about 65 mg/L of bromide ion. If the NaCl used contained some bromide ion, then when treated with ozone, the bromide ion would be oxidized to hypobromous acid (HOBr). If the method for determining dissolved "ozone" was based on iodometry (oxidation of iodide ion to free iodine), both the ozone and any HOBr produced during ozone oxidation of bromide ion would read as "dissolved ozone". Consequently, the petitioners suggest ignoring data in this last column of Table 2.3.11.

Table 2.3.11. Ozone Concentrations in Water After Flushing at Different Conditions (Chen et al., 1987)

Flushing time, min	0EC water	5EC water	15EC water	25EC water	0EC water + 300 ppm milk	0EC water + 0.3% NaCl
0	0	0	0	0	0	0
2	0.95	0.91	0.66	0.32	0.32	2.05
4	1.37	1.24	0.97	0.48	0.53	2.21
6	1.48	1.24	1.02	0.53	0.74	3.78
8	1.58	1.49	1.02	0.58	0.90	4.05
10	1.79	1.49	1.07	0.58	1.11	4.15
12	1.74	1.49	1.02		1.11	4.10
14	1.74				1.69	4.05
16	1.63				1.95	4.15
18					2.27	4.26
20					2.21	4.20
A Distilled Water.						

4. Figure 2.3.04 shows the ozone concentrations in distilled water at the four temperatures tested.

5. Figures 2.3.04 through 2.3.08 show microbiocidal effects of ozone on the various microorganisms tested by Chen et al., 1987.

2.3.5 Chen et al. (1992) – Shrimp Meat Extracts

Abstract: We examined solubility and stability of ozone in shrimp-meat extract (SME), bactericidal effect of ozone on shrimp-meat microorganisms, mutagenicity of ozonated shrimp meat, and ozonolysis of DNA. The saturated concentration (1.4 mg O_3/L) of ozone in SME was lower than in 2% saline or distilled water at 5 and 25EC. Upon standing for 25 min after ozonation, ozone exhibited the same decomposition rate (2.7%/min) in 5 and 25EC SME. Among 9 bacterial strains tested, *Salmonella typhimurium* was more resistant to ozone in shrimp meat. Mutagen was not detected in shrimp meat when it was ozonated in saline. Ozone in saline (less than 5 mg O_3/L) could lyse M13 RF DNA in *Escherichia coli* and single-stranded DNA in phage M13 outside the bacterial cell within 30 min.

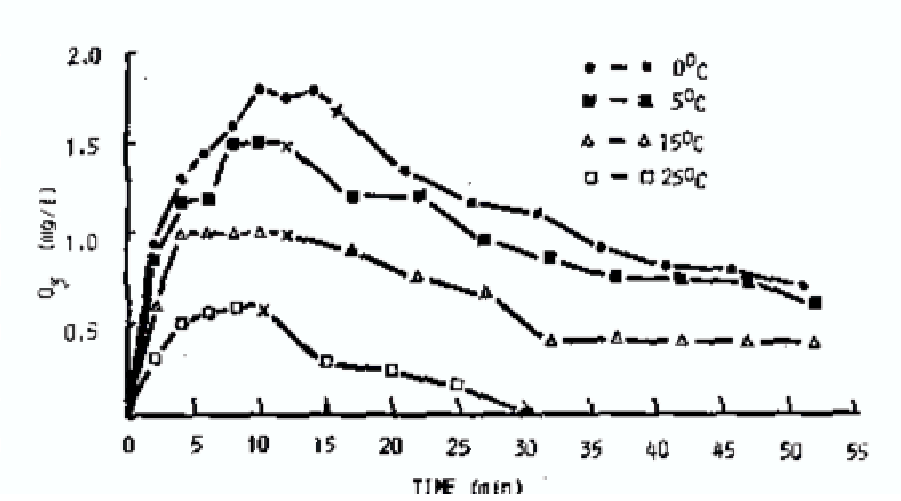

Figure 2.3.04. Ozone concentrations in distilled water at different temperatures during and after flushing with ozone. "X" indicates the time when ozone flushing was ceased (Chen et al., 1987).

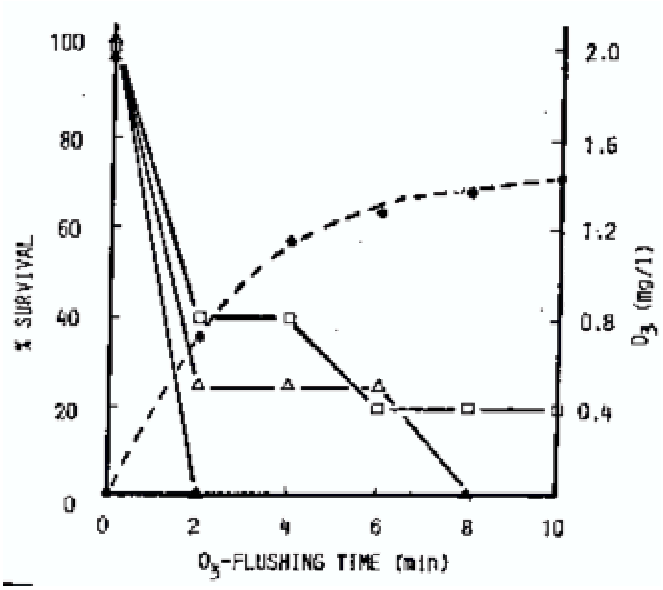

Figure 2.3.05. Sterilization effect of ozone-treated distilled water at 5EC on *Vibrio cholerae, Escherichia coli, Salmonella typhimurium* (>), *Vibrio parahaemolyticus* (♠) and *Staphylococcus aureus* (9). Ozone concentration indicated as (X) (Chen et al., 1987).

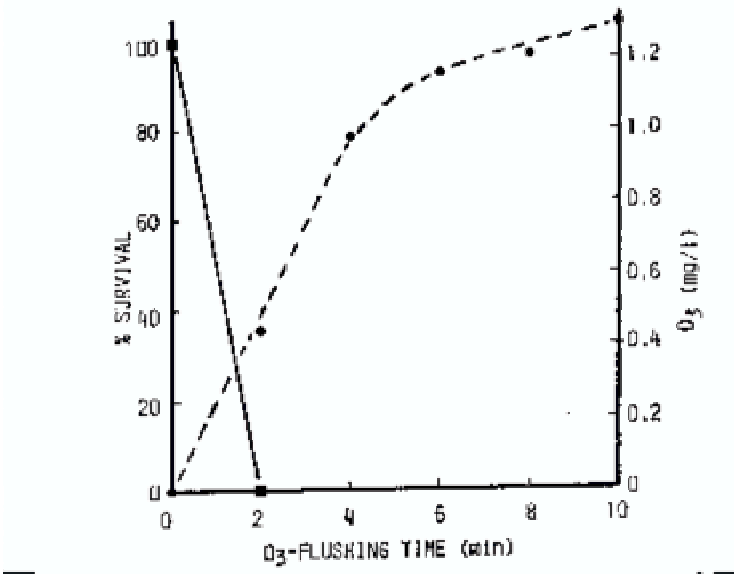

Figure 2.3.06. Sterilization effect of ozone-treated distilled water at 25EC on *Vibrio cholerae, Escherichia coli, Salmonella typhimurium, S. aureus* ( ). Ozone concentration indicated as (X) (Chen et al., 1987).

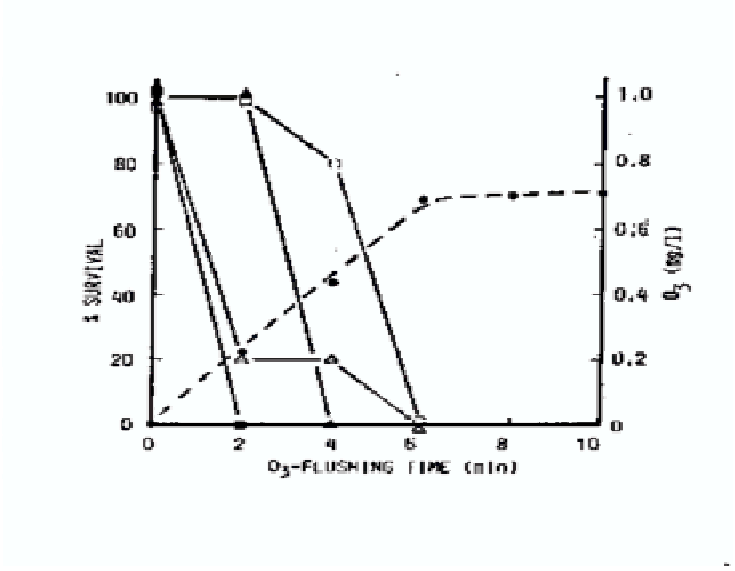

Figure 2.3.07. Sterilization effect of ozone-treated water mixed with 300 ppm milk powder at 5EC on *Vibrio cholerae* (♠), *Vibrio parahaemolyticus*, *Staphylococcus aureus* ( ), *Escherichia coli* (~), and *Salmonella typhimurium* (>). Ozone concentration indicated as (X) (Chen et al., 1987).

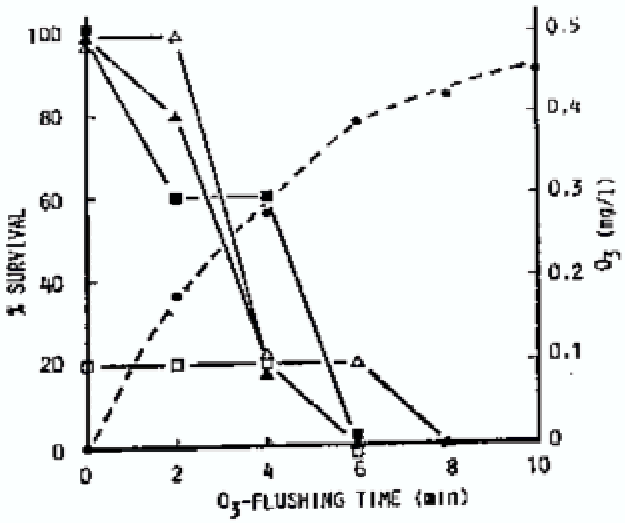

Figure 2.3.08. Sterilization effect of ozone-treated water mixed with 300 ppm milk powder at 25EC on *Vibrio cholera*, *Salmonella typhimurium* (>), *Escherichia coli* (♠), *Vibrio parahaemolyticus* (9),and *Staphylococcus aureus* ( ). Ozone concentration indicated as (X) (Chen et al., 1987).

The objective of this work was to study the solubility and stability of ozone in liquid phase, and the effect of ozone on the inactivation of some bacterial strains suspended in saline or seeded in shrimp meat. The formation of mutagens had been observed in shrimp meat which had been soaked with ozone flushing, and the ozonolysis of DNA in phages and bacteria when they were treated in ozonated water with concentrations used for disinfection.

Materials and Methods

Microorganisms: Nine bacterial strains were used to test the bactericidal effects of ozone. They included *Pseudomonas aeruginosa* CCRC 10261, *P. fluorescens* CCRC 10304, *P. putida* CCRC 10459, *Escherichia coli* CCRC 11634, *Flavobacterium aquatile* ATCC 11947, *Salmonella typhimurium* ATCC 15277, *Staphylococcus aureus* ATCC 6538p, *Vibrio parahaemolyticus* 10145, and *Vibrio cholerae* Inaba. For mutagenicity tests, *Salmonella typhimurium* TA98, TA100, and TA102; for tests of ozonolysis of DNA, *E. coli* JM107 and phage M13 were employed.

Microbial Assay: Aerobic plate count (APC) was determined by using plate count agar (PCA, Difco) which was incubated at 25 or 35EC for 48 hr. For enumeration of bacterial strains suspended in saline or seeded in shrimp meat, selective or non-selective media were used (*E. coli* : deoxycholate agar, Difco; *S. typhimurium*: xylose lysine desoxycholate agar, Difco; *S. aureus*: Baird-Parker medium, Difco; *V. parahaemolyticus* or *V. cholerae*: thiosulfate-citrate-bile salts-sucrose agar, Difco; *P. putida* or *P. fluorescens*: pseudomonas isolation agar, Difco; *P.*

aeruginosa: pseudomonas F agar, Difco; *F. aquatile*: pale yellow-brown colony on tryptic soy agar, Difco). Except *P. fluorescens, P. putida* and *F. aquatile* were incubated at 25EC, all other bacterial strains were incubated at 35EC for 48 hr. Since background (or original) APCs (proximate 10^3 CFU/g) of shrimp meat were much lower than the counts (proximate 10^6 CFU/g) after seeding with bacteria, further confirmation of typical colonies on selective or non-selective media was not conducted.

Stability and Bactericidal Effects: Solubility and stability of ozone in distilled water (pH 6.2), 2% NaCl solution (saline, pH 7.0) (Petitioners' Note: see Note 3 in Chen et al., 1987), and 1% shrimp meat extract (SME, pH 7.8) were examined. SME was prepared by boiling 1 part (by weight) of peeled tiger prawn (*Penaeus monodon*) meat with 99 parts of distilled water for 30 min, followed by filtering through cheese cloth and Whatman No. 1 filter paper.

From each liquid 4L were equilibrated al 25 or 5EC for 1 hr prior to flushing with ozone (100 mL/min). During flushing, each liquid was kept in a water bath of desired temperature. Liquid (100 mL) was removed and ozone concentration was detected by using the iodometric titration method (**Petitioners' Note: see Note 3 in Chen et al., 1987**) at first 1 min, and then 2 min intervals, until the liquids were saturated with ozone. However, flushing was stopped for 30 sec during each liquid-removal operation. When the concentrations of ozone in liquids became stable (or saturated), flushing was terminated. And subsequently, ozone concentration in the liquid was detected at 5 min intervals to examine the decomposition of ozone in the liquids until 25 min.

For detecting the bactericidal effect of ozone on 9 bacterial strains in 0.8% saline, the tested strains were inoculated in nutrient broth (NB, Difco) for activation. The colony-forming units (CFU) of the tested strains in NB were enumerated by plating bacteria on PCA, and incubating at 25 or 35EC for 48 hr. T he bacteria in NB were diluted using sterile phosphate buffer solution to obtain the suspensions containing the cell counts of 10^6-10^8 CFU/mL A portion of bacterial suspension was poured into 4-L of 0.8% saline (25EC) for the cell counts of 10^5-10^7 CFU/mL. The bacteria-containing saline then was dispersed with ozone (100 mL/min) for 1 min. Immediately after flushing stopped, 1 loopful of ozonated suspension was transferred to a test tube containing 5 mL of NB. Ten tubes for each bacterial strain were transferred. Ozone concentration in the suspension was detected at the same time. During removal of liquid for ozone concentration detection, the flushing operation was stopped for 1 min. After that, each 2 min, the flushed suspension was transferred into NB-containing tubes and ozone concentrations in the suspension also were detected following the procedure above. The tubes inoculated with flushed suspension were incubated at 25 or 35EC for 48 hr to examine growth of bacteria. Percent survival of each bacterial strain after treatment was calculated from the ratio of the number of tubes indicating growth to the number of tubes tested (i.e., 10 in this test).

For detecting the effect of ozone in 2% saline on the disinfection of 9 bacterial strains seeded in shrimp meat, 200 g of shrimp meat was washed with sterile water and then dripped dry for 5 min. Immediately after dripping, 15 mL bacterial suspension (10^6-10^8 CFU/mL) was mixed thoroughly with shrimp meat and then dripped dry again for 5 min. For enumeration of original APC of whole shrimp or shrimp meat, washing and bacteria-seeding operations were omitted. The bacterial counts in bacteria-seeded shrimp meat (25 g) were enumerated using selective or non-selective media, before soaking in ozonated saline. The remaining bacteria-seeded shrimp

meat (175 g) was immersed in 4-L 2% saline (5EC) which had been flushed with ozone (150 mL/min) for 30 min before the meat was soaked. Ozone concentration in the saline was measured before immersion. At 15 min intervals, 25 g of shrimp meat was removed for enumerating bacterial counts by using selective or non-selective media until 60 min. Ozone concentration in saline also was detected in every period of meat removal. Ozone was dispersed into the saline throughout the test, except during the removal of saline for the test of ozone concentration. A control test (duplicate) flushing with air (150 mL/min) to obtain the same stirring effect also was conducted.

Mutagenicity Test of Ozonated Shrimp Meat Extract: Shrimp meat (200 g) was washed with distilled water, and then immersed in ozonated 2% saline (5.2 mg-ozone/L, 5EC. Ozone was flushed (150 mL/min) until termination (120 min) of the test (final ozone concentration 5.1 mg/L). Every 30 min, 25 g of immersed meat was removed and dripped dry 5 min, and then homogenized with 150 mL of distilled water. The homogenate then was filtered through cheesecloth. Acetone was added to the filtrate to make up 65% of acetone concentration. The suspension was filtered again by using Whatman No. 1 and No. 42 filter papers. Acetone was removed from the filtrate by use of a rotary evaporator at 32EC. The residue was adjusted to pH 10 using 10% ammonium water. The resultant solution was extracted two times using 150 mL dichloromethane. The water layer was collected and condensed (at 35EC) to 3 mL and then frozen for further treatment. The volume of dichloromethane-extracted liquid was reduced in a rotary evaporator to 0.2 mL and then washed out with 4.8 mL of dimethylsulfoxide (DMSO.

For the water layer, histidine was decarboxylated to form histamine. The pH of the water layer was adjusted to 4.5 by using 0.2M sodium acetate buffer. To 10 mg of histidine in the sample, 2 mg of histidine decarboxylase (0.33 unit/mg) was added. After 2 hr incubation at 37EC, the solution was centrifuged (12,000 X g, 20 min) to eliminate the enzyme in suspension and then filtered through a 0.22 Φm membrane. Filtrate (2 mL) was kept at -20EC. Completion of decarboxylation was determined by observing the disappearance of histidine and its derivatives after separation by paper electrophoresis.

The mutagenicity test with *Salmonella typhimurium strain* TA98, TA100 and TA102 was performed following the procedure described by Maron and Ames (1983). When a plate incorporation test was carried out, 0.1 or 0.3 mL of DMSO-dissolved extract, or 10 or 40 ΦL of condensed and histidine-removed water soluble extract was added to each plate. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used as a positive control for strains TA100 and TA102; while nitro-o-phenylenediamine (NPD) was used for strain TA98.

Results and Discussion

Solubility and Stability of Ozone in Shrimp Meat Extract: Fish and shellfish meats contain water soluble substances which are mostly organic matter. When the meat is washed or immersed in water, accordingly, such substances will be dissolved in water, and may interfere with the solubility of ozone in water, if the water is ozonated. Shrimp meat extract (SME) was used instead of effluent from marine food industries.

Solubility and stability of ozone in SME compared with distilled water and 2% NaCl solution at 5 or 25EC are shown in Figures 2.3.09 and 2.3.10, respectively. At a given flow rate (100

mL/min) and at 5EC, three liquids were saturated with ozone within 27 to 35 min. The saturated concentration of ozone in SME was 1.4 mg/L, which was 3.8 or 2.8 times lower than that in 2% saline or distilled water at 5EC. In contrast, at 25EC, the saturated concentrations in three liquids were lower than at 5EC; distilled water, 2% saline and SME were ozone saturated in concentrations of 2.3, 2.8, and 0.6 mg/L Ozone solubility was higher in 5EC than in 25EC water, 2% saline or SME.

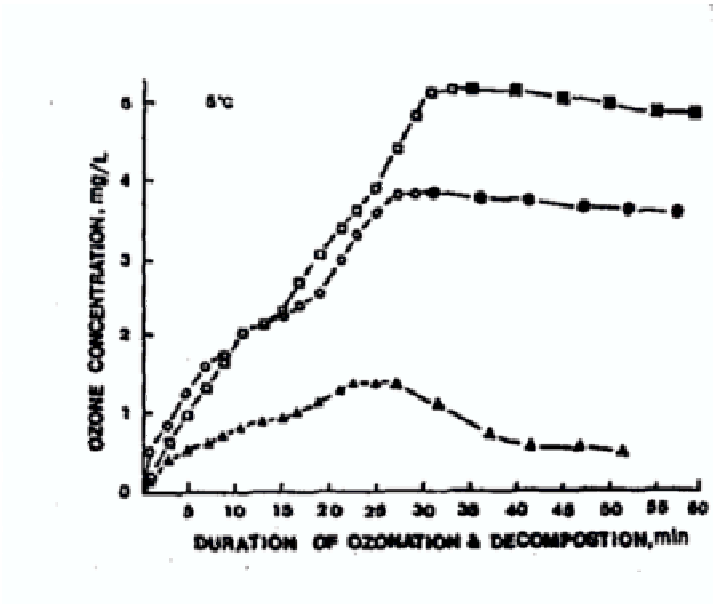

Figure 2.3.09. Solubility and decomposition of ozone in distilled water (", !), 2% NaCl solution (G, #) and shrimp meat extract (♠, >), when the 5EC liquids were ozonated at a flow rate 100 mL/min. Solid marks indicated ozone flushing was stopped and ozone was decomposed.

Bactericidal Effect: Inactivation of microorganisms suspended in 0.8% saline (25EC) by ozone is shown in Table 2.3.12. *V. cholerae* (10^7-10^8 CFU/mL) was completely destroyed by ozone in a final concentration of 0.95 mg/mL when ozone had been flushed for 17 min at a flow rate of 100 mL/min. Among 9 bacterial strains, *S. aureus* was more sensitive to ozone, while *F. aquatile*, which maintained 20% survival after ozonating for 27 min, was more resistant to ozone. This could be because our cells were suspended in saline (0.8% NaCl) and microorganism destruction by ozone was less effective in Ringer solution than in distilled water (Yang and Chen, 1979).

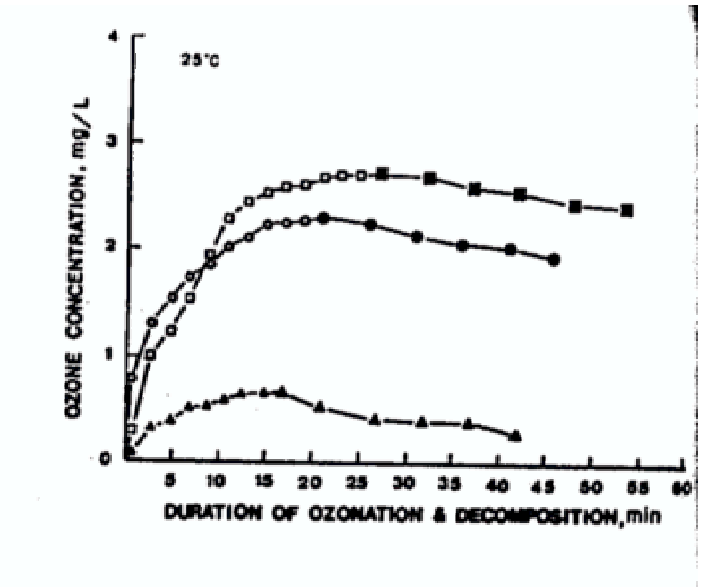

Figure 2.3.10. Solubility and decomposition of ozone in distilled water (", !), 2% NaCl solution (G, #) and shrimp meat extract (♠, >), when the 25EC liquids were ozonated at a flow rate 100 mL/min. Solid marks indicated ozone flushing was stopped and ozone was decomposed.

Destruction of bacteria inoculated in shrimp meats which were immersed in ozonated 2% saline (5EC) is shown in Table 2.3.13. Saline (2%) was flushed with ozone (150 mL/min) to obtain concentrations from 2.9 to 4.8 mg-ozone/L in each treatment. When 175 g bacteria-seeded shrimp meat was immersed in 4-L ozonated 2% saline, ozone concentration reduced more than 1.4 mg/L within 15 min. Since ozone was flushed continuously during soaking (except during removal of liquid), the ozone concentration in saline increased gradually after decreasing (data not shown). A portion of bacteria seeded on shrimp meat, immersed in each bacterial suspension before flushing with air or ozone, could be washed off. Thus, reduction of bacterial count during the first 15 min of flushing treatment was obvious. Therefore if the survival of each seeded bacterial strain after 15- to 60-min flushing is considered, *F. aquatile* in shrimp meat was more sensitive to ozone, since its population reduced 0.89 log cycle during ozonation when final ozone concentration was 2.35 mg/L.

S. typhimurium was more resistant to ozone (reduced only 0.1 log cycle) when the final ozone concentration was 3.02 mg/L. Among 9 bacterial strains tested, *F. aquatile* was more resistant to ozone in 0.8% saline (Table 2.3.12), but was more sensitive to ozone in shrimp meat. This discrepancy remains to be investigated. Saline (2%) without ozonation had a protective effect on all bacterial strains inoculated in shrimp meat, except *S. typhimurium*. After soaking in ozonated 2% saline for 60 min, reduction of APC in whole shrimp was more than 1.27 log cycles; while in shrimp meat, the reduction was more than 0.54 log cycle. However, it was impossible to sterilize a grass prawn (85 g) or a piece of shrimp meat (45 g) by soaking them in an ozonated 2% saline (5 mg-ozone/L) less than 60 min.

Table 2.3.12. Bactericidal effect of ozone on microorganisms suspended in 25EC 0.8% saline [a] (Chen et al., 1992)

Microorganism	Inhibition [b] time, min	Final O_3 concentration showing inhibition, mg/L
Vibrio cholerae	17	0.95
V. parahaemolyticus	13	0.81
Flavobacterium aquatile	> 27 [c]	1.00
Pseudomonas aeruginosa	9	0.34
P. putida	11	0.78
P. fluoroscens	13	1.07
Escherichia coli	9	0.50
Salmonella typhimurium	17	0.54
Staphylococcus aureus	5	0.3

a Microorganisms were seeded to 10^5-10^7 CFU/mL suspension, prior to ozonating. Flow rate of ozone was 100 mL/min.

b Time needed to show 100% inhibition obtained from the ratio of the number of tubes indicating no growth to the number of tubes tested (10 in this test).

c Only 80% of *F. aquatile* was inhibited within 27 min, while ozone concentration was 1 mg/L.

Saline (2%) was used because concentration of saline below 2.5% NaCl enhances the bactericidal effect of ozone, while 5% NaCl showed a slight protective effect (Yang and Chen, 1979). In addition, 2-3% NaCl-containing saline can change the structure of the meat surface, thus reducing the attachment of bacteria to the meat surface. Ozonated saline cannot effectively permeate into shrimp meat. A low concentration of ozone is ineffective for disinfection when organic matter is present to interfere with the action on bacterial cell. Thus, the bactericidal effect of ozone applied for shrimp-meat disinfection was not efficient. Haraguchi et al. (1969) soaked gutted fish in 3% NaCl solution containing 0.6 ppm ozone for 30-60 min, resulting in a 4-5 log-cycle reduction of viable bacterial counts on skin of the fish. However, they could not sterilize the fish by using ozone.

Food Safety (Mutagenicity) Considerations: Ozone has long been known as a powerful oxidative agent. Menzel (1984) postulated that ozone may develop toxic substance in foods because of the oxidation of tissue proteins or unsaturated fatty acids in foods. Shrimp meat contains high level of proteins and small amount of fats (Martin et al., 1982). The Ames test was conducted to examine the formation of mutagens in both fat and water-soluble extracts of shrimp meat after ozonating in 2% saline. Immersion of shrimp meat in saline containing 5 mg-ozone/L, for 120 min, did not induce mutagens in shrimp meat (Table 2.3.14). The reduction of revertants on the plates to which 0.3 mL lipid soluble extract was added, could be due to the toxic effect of DMSO.

Table 2.3.13. Bactericidal effect of ozone on the microorganisms inoculated on shrimp meat which was immersed in 5EC ozonated [a] 2% saline (Chen et al., 1992)

Microorganism	Ozone concentration (mg/L) during treatment (min)			Microorganism survival (log CFU/mL) during treatment (min)			
	0	15	60	0	15	60	Difference [b]
Escherichia coli	4.45	2.41	3.54	6.50 (5.52) [c]	4.51 (5.31)	4.31 (5.31)	-0.20 (0.08)
Flavobacterium aquatile	4.78	1.25	2.35	5.48 (5.86)	4.61 (5.52)	3.72 (5.61)	-0.89 (0.09)
Pseudomonas aeruginosa	3.48	1.75	2.98	6.02 (6.52)	5.43 (6.49)	4.85 (6.54)	-0.58 (0.05)
P. pituda	4.09	2.41	3.37	5.29 (5.41)	4.44 (5.11)	4.19 (5.10)	-0.25 (-0.01)
P. fluorescens	3.92	1.78	3.27	7.00 (6.85)	6.21 (6.32)	5.51 (6.33)	-0.70 (0.01)
Salmonella typhimurium	3.29	1.85	3.02	7.59 (6.62)	7.50 (6.36)	7.40 (6.30)	-0.10 (-0.06)
Staphylococcus aureus	3.41	1.59	3.25	6.55 (5.54)	5.32 (5.20)	4.82 (5.23)	-0.50 (0.03)
Vibrio cholerae	4.25	1.93	3.83	7.23 (6.63)	6.92 (6.28)	6.50 (6.26)	-0.42 (-0.02)
V. parahaemolyticus	3.21	1.48	3.02	6.39 (5.93)	5.91 (5.54)	5.30 (5.55)	-0.61 (0.01)
APC of shrimp meat [d]	2.94	2.75	3.55	3.20 (3.54)	2.94 (3.31)	2.40 (3.28)	-0.54 (-0.03)
AOC of whole shrimp	3.75	2.94	4.43	3.75 (3.86)	3.60 (3.56)	2.39 (3.54)	-1.21 (-0.02)

a Ozonation proceeded during the test at a flow rate of 150 mL/min.
b Value obtained in 60 min after flushing was subtracted from that in 15 min.
c Value in parenthesis was the result of flushing with air.
d APC = aerobic plate count.

Effect of Ozone on M13 Phage ssDNA and RF DNA: DNA of phage M13 suspended in M9 medium is single-stranded (ssDNA). When M13 is transformed into *E. coli* JM107, the DNA of phage M13 in *E. coli* JM107 is double-stranded (RF DNA). It is hypothesized that ssDNA will be cleaved into segments or linear form DNA, and double-stranded supercoil DNA (scDNA) will be conversed to open circular DNA (ocDNA) or linear form, when ssDNA or scDNA are treated with ozone.

Table 2.3.14. Mutagenic response of lipid and water-soluble fractions of shrimp meat immersed in 5EC ozonated 2% saline containing 5.2 mg-ozone/L (Chen et al., 1992)

Ozonating time or chemical added	Revertants/plate					
	TA98		TA100		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9
Blank [a]	25	32	106	110	229	310
Ozonating time (min) – lipid soluble fraction [b]						
0	23(9)	35 (23)	104 (75)	113 (83)	198 (110)	296 (291)
30	18 (14)	28 (14)	105 (80)	112 (80)	292 (102)	373 (206)
60	18(10)	30 (17)	89 (78)	90 (83)	214 (187)	328 (312)
90	20 (11)	28 (20)	97 (80)	105 (86)	180 (129)	320 (298)
120	16 (9)	32 (20)	83 (76)	107 (89)	222 (109)	317 (219)
Water soluble fraction [c]						
0	23 (12)	35 (17)	91 (76)	90 (54)	196 (110)	396 (194)
30	15 (19)	28 (32)	82 (54)	72 (62)	198 (96)	284 (238)
60	21 (22)	33 (30)	74 (52)	81 (49)	116 (116)	204 (250)
90	23 (13)	28 (39)	64 (46)	77 (55)	150 (123)	208 (184)
120	15 (18)	27 (27)	66 (38)	66 (35)	66 (91)	172 (133)
Control [d]						
DMSO, 0.1 mL/plate	20	31	106	101	230	308
NPD, 20 Φg/plate	1550	1810				
MNNG, 2.0 Φg/plate			10100	9340	7110	6170

a For spontaneous reversion.
b To each plate, 0.1 mL DMSO-dissolved extract, or 0.5 g-equivalent amount of ozonated shrimp meat was added; revertants inside parentheses indicated the results that 0.3 mL DMSO-dissolved extract, or 1.5 g-equivalent of ozonated shrimp meat was added to each plate.
c To each plate, 10 ΦL of condensed and histidine-removed extract, or 0.13 g-equivalent amount of ozonated shrimp meat was added; revertants inside parentheses indicated the results that 40 ΦL of condensed and histidine-removed extract, or 0.5 g-equivalent of ozonated shrimp meat was added to each plate.
d DMSO (dimethylsulfoxide) was used as negative control; NPD (4-nitro-o-phenylenediamine) and MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) were used as positive control.

In our test, survival of *E. coli* JM107 and phage M13 treated by ozonating (100 mL/min), final ozone concentration was 5 mg/L) is shown in Figure 2.3.11. The bacterial count or phage titer reduced more than 8 or 5 log cycles, respectively, after 30-min ozone flushing. During ozonation (less than 5 mg-ozone/L), M13 RF DNA (scDNA) did not convert to ocDNA.

However, three segments of M13 RF DNA might be cleaved into shorter segments which were too small to be observed on agarose gel after 30-min ozonation. Likewise, extensive shearing of M13 ssDNA outside bacterial cells occurred when the DNA was ozonated more than 25 min, since few DNA fragments were clearly observed on agarose gel. This may be one reason that ozone exhibited bactericidal effects.

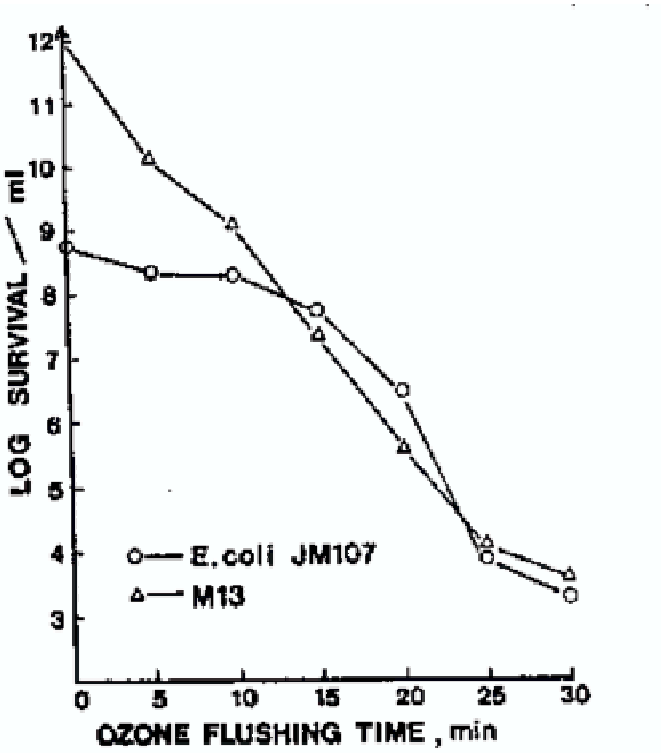

Figure 2.3.11. Effect of ozonation on the survival of E. coli JM107 and phage M13 in M9 medium which was ozonated with a flow rate of 100 mL/min, to obtain a final concentration of 5 mg-ozone/mL within 30 min. (Chen et al., 1992).

2.3.6 Blogoslawski et al. (1993) – Shrimp Mariculture

Abstract: During experiments from April 1990 to May 1991 ozone gas was reacted with seawater to reduce levels of disease-causing *Vibrio* bacteria at a shrimp hatchery, LARFICO, located near Ayangue, Ecuador. A 1,540 liter capacity fiberglass contact tower having a five to seven minute retention time treated Pacific Ocean seawater for an ozone-produced oxidant residual average of 0.07 mg/liter. From February 1991 to May 1991. ozonized seawater was used in experimental larval tanks of 13,000 liter capacity while similar tanks acted as controls. Since ozone eliminated *Vibrio* as determined by TCBS plating of treated water while control water showed *Vibrio* colonies too numerous to count which caused shrimp to die of disease, the entire hatchery (30 tanks of 13,000 liters each) was treated with ozonized seawater from June 1991 to September 1992.

In addition to elimination of Vibriosis which caused disease, ozonized seawater was shown to decrease the time required for normal molting and to reduce the total growth cycle by three days

versus control water without ozone treatment. In one year of operation using ozone, survival rates of larval shrimp were robust, antibiotic use was reduced, and one additional growth cycle was realized.

Materials and Methods

Ozone Contacting: Raw seawater was pumped to a main cistern where it was heat- and UV-treated prior to being pumped to the ozone treatment area at a rate of 40-60 gpm depending on experimental. and later, commercial requirements. Two methods of contact were designed and constructed to introduce ozone to the seawater: (1) a Mazzei venturi injector system (2 inch bypass type) was installed which permitted ozone to be introduced directly into the piping carrying the water from the main cistern; and (2) a contacting tower with a volume of 1540 liters was constructed of fiberglass with three large air stones at the base. The tower was filled with seawater from the main cistern and ozone was bubbled into this water through the air stones. Complete turnover in the tower was adjusted between five and seven minutes and the flow rate varied according to the demands of the hatchery system.

Ozonized seawater at average ozone doses of 0.07-0.08 ppm was sent to all larval tanks to control Vibriosis.

"Ozone" Residual: When dealing with a seawater system, the residual measured is actually an ozone produced oxidant (OPO) rather than ozone due to the ready combination of ozone with various chemical species found in seawater, particularly bromide ion. Therefore, the value reported reflects the OPO rather than a true ozone residual. Water samples for the determination of oxidant residuals were obtained by immersing a 250 mL graduated cylinder in the water source. The sample was then brought to the Bacteriology Laboratory where residual determinations were made using the 2% KI-sodium thiosulfate titration method.

LARVAL SYSTEM: Larval Tanks: The larval tanks are rectangular and are constructed of concrete. They have a total volume of 13,000 liters and are kept in bright warm rooms. Water temperature in the tanks ranged from 27 to 31EC throughout the study period. Water exchanges with ozonized water were performed on a regularly scheduled basis. Thus, the tanks were filled with ozonized seawater which then stood in the tanks until the tanks were partially drained at a scheduled time and the water replaced with freshly ozone-treated water.

Larval Shrimp: Nauplii of *Penaeus vannameii* are supplied from the wild or from nauplii secured from the maturation system at LARFICO. Nauplii from both sources were used throughout the study period but were always seeded into separate tanks.

Larval Shrimp Food and Tank Additives: Algae, including *Chaetoceros gracilis* are added to the larval tanks to serve as a food source for the shrimp. *Artemia* also serve as a primary food for the shrimp larvae. Prior to the full-scale implementation of ozone, artificial foods, EDTA, copper, formalin, Treflan, and antibiotics also were added to the tanks.

BACTERIA STUDIES: Water Samples for Bacterial Counts: A 12 x 75 mm sterile tube was immersed in the tank and capped upon removal. The tube of water then was transported to the

Bacteriology Laboratory and plated immediately upon selected media: TCBS for *Vibrio* isolation and OZR for total marine bacteria counts.

Bacteria Counts: Samples were plated upon TCBS agar in all studies and upon TCBS and OZR in selected studies. Samples were plated according to the following protocol: 0.1 mL by sterile pipette, spread in a three quadrant method; ten microliter by sterile loop, plated in a three quadrant method; and 0.1 microliter by sterile loop, plated in a three quadrant method. All plates were incubated at room temperature (24-26EC) and read after approximately 18 hours.

Bacterial Studies: Bacterial studies were conducted on the following:

- Water from the maturation tanks
- Shrimp nauplii from the maturation tanks and from the wild
- Water from the larval rearing tanks
- Algae used as food for shrimp larvae
- *Artemia*, a commercially acquired food source for shrimp larvae
- Commercially prepared foods used for shrimp larvae
- Various environmental cultures of the physical plant and water system

RESULTS AND DISCUSSION

The results and discussion section of this paper will consider the following areas separately: Experimental Studies and Commercial Production Studies.

EXPERIMENTAL STUDIES: Maturation Tanks: Two tanks were subjected to ozone treatment to determine the ability of ozone to reduce Vibriosis in the maturation system. Prior to ozone treatment, the tanks yielded *Vibrio* counts of greater than 200 colonies on TCBS agar at a concentration of 10^{-2}. *Vibrio* counts were reduced by approximately one-half every three hours after the start of ozone treatment, reaching zero between three and six hours after the initiation of ozone treatment. The treatment did not appear to affect the shrimp adversely as the animals continued to display normal movement throughout and after the ozone process. There was no mortality of shrimp during or 24 hours after the experiment.

Foods: Six artificial foods used in rearing larval shrimp were plated for *Vibrio*. Only one of the foods grew out *Vibrio* at a significant level. Algae used as food for larval shrimp were plated on TCBS. Zero plate counts indicated that the algae were not a source of the Vibriosis experienced at the hatchery.

Artentia: Prior to addition to ozonized water in a larvae tank, the *Artemia* examined showed a count of 350 *Vibrio* colonies at a concentration of 10^{-1}. Vibrio counts were significantly reduced to 21 colonies at 10^{-1} ten minutes after the *Artemia* were exposed to ozonized water. After 50

minutes in the tank with ozonized water, the *Vibrio* counts from the *Artemia* were reduced to 3 colonies at 10^{-1}.

Shrimp Nauplii: Unwashed nauplii from the wild contained high *Vibrio* concentrations as indicated by elevated plate counts on TCBS.

Larval Tank Studies: All preliminary studies showed that ozone-treated tanks experienced reduction in *Vibrio* counts as opposed to tanks not exposed to ozone treatment. Neither the water nor the algae added to the tanks contained any measurable *Vibrio* prior to addition to the tanks. Nauplii from wild seed, *Artemia*, and one artificial supplemental food used did produce *Vibrio* counts. When any of these were added to the tanks, the *Vibrio* counts increased dramatically. With exposure to ozone-treated water, however, the counts fell back to low or non-detectable levels and disappeared after a fresh exchange occurred in the tank with ozonized water. The following example is typical of the larval tank experiences:

After nauplii were added to a tank at 13:00, the plate count for *Vibrio* rose to 9 colonies at 10^{-1} on TCBS. Three hours later, the count fell to one colony. At midnight, after food which contained *Vibrio* had been added to the tank, the plate count rose to 32 colonies at 10^{-1}. At 9:45 the following morning, the *Vibrio* count read 30 colonies on TCBS at 10^{-1}. After ozonized water (0.17 ppm OPO) was added to the tank at 10:30, the *Vibrio* count fell to zero and remained there. By 15:45, no OPO was detected in the tank, indicating the high oxidant demand of the bacteria, excess algae, and moribund larvae. The count at 17:30 showed approximately 200 colonies of *Vibrio* on TCBS at 10^{-1}. By 9:30 the next morning, however, the *Vibrio* level had become too numerous to count. Ozonized water (0.48 ppm at 10:30 and 0.22 ppm at 11:45) was added to the tank at 10:00 and the *Vibrio* count was recorded as zero at 10:30.

The ozone-produced residual in the tanks appeared adequate for the most part to reduce *Vibrio* counts throughout the study period. In contrast, tanks without ozone treatment required the addition of several antibiotics to carry the larvae through. The antibiotics used included: Chloramphenicol, Erythromycin, Neomycin, and Tetracycline. Further disinfectants added to the non-ozone tanks included EDTA, Treflan, copper, and formalin.

Thus, the preliminary experiments indicated that:

- A Vibriosis was responsible for the mortality experienced at the hatchery
- The source of the *Vibrio* could be traced to *Artemia*, to nauplii from wild stock and to an artificial supplemental food given to the larvae
- Ozone-treated seawater appeared to be quite successful in reducing *Vibrio* concentrations in the larval tanks.

Selected tanks were treated with ozonized seawater throughout the entire larval cycle. During these studies it was observed that when ozone-treated water contained a residual exceeding 0.1 ppm, the shrimp larvae appeared markedly affected. The larvae sank to the bottom of the tank and exhibited damaged appendages upon microscopic examination. After a few hours, however, the same larvae were observed to molt and exhibit normal behavior in spite of the damaged

appendages. The larvae rose to the surface, swam and fed actively. This observation led to the speculation that careful manipulation of the oxidant residual could have the dual benefit of controlling the *Vibrio* concentration and, by induction of' molting, shorten the larval growth period. In addition, the ozone-treated tanks continued to require far fewer antibiotic treatments than the control tanks that did not receive ozonized seawater. Due to the continued success of the ozone-treated tanks, the decision was made to treat the entire 30 tank larval rearing system with ozonized seawater.

COMMERCIAL PRODUCTION STUDIES: Thirteen complete cycles of larval production have been accomplished to date using ozone throughout the entire larval rearing system at LARFICO. In addition to supplying ozone-treated water for all exchanges of water for the larval tanks, ozone also is used to supply washes for nauplii and *Artemia* prior to the addition of those species to the larval tanks.

The ozone produced residual is regulated throughout the cycle so that it ranges from 0.066 to a high of 0.250 ppm.

The residuals noted above have proved to be effective in controlling bacterial populations, including *Vibrio sp.* Counts on TCBS range from a high of 690 colonies at $l0^{-2}$ to zero following a water exchange when a portion of the water is drained from the larval tanks and is replaced with freshly-treated ozonized water.

The use of ozone-dosed seawater producing residuals above 0.1 ppm has consistently caused early molting in the larvae, resulting in an average reduction of three days in the total growth cycle. This has permitted an additional rearing cycle during the year that ozone has been used in all thirty tanks.

The percent survival of larvae during the period of ozone use has ranged from 60.0 to 99.1%, indicating that the oxidant has not diminished larval survival. Interestingly, the cycle with the highest percent survival occurred when the highest oxidant residuals were recorded.

Finally, the use of ozone was found to reduce or eliminate several additives formerly used in the tanks to enhance larval survival. Those substances completely eliminated since the implementation of ozone treatment include EDTA, copper, formalin, Treflan, and all supplementary feeds. Importantly, the use of antibiotics has been reduced by nearly 2/3 from 300 kg/year before ozone to approximately 100 kg/year with ozone treatment.

CONCLUSIONS: Preliminary studies indicate that ozone could be used effectively in the maturation system to reduce levels of bacterial pathogens from that system without causing harm to the adult shrimp.

Ozone washes have proved effective in reducing levels of bacteria pathogenic to shrimp larvae from larval foods such as *Artemia*.

It has been demonstrated that seawater treated with ozone is able to reduce levels of or eliminate bacteria pathogenic to shrimp larvae without damaging the larvae if the ozone-produced residual is kept below 0.2 ppm, the average residual for this study being 0.07 ppm.

The most efficient method of contacting ozone and seawater for this hatchery proved to be a contacting tower which allows a five to seven minute retention time.

Ozone treatment was found to speed the molting process, permitting a reduction of an average of three days from the larval cycle. When expanded throughout the entire larval rearing system, these extra days allowed an additional rearing cycle over the course of the first year of ozone use resulting in increased annual production and revenue. Prior to the use of ozone, annual production from 1988-1991 averaged 120,000,000 larvae at LARFICO. With ozone, the annual production figure for larvae has increased to approximately 260,000,000, a doubling of hatchery output.

2.3.7 Arimoto et al. (1996) – Striped Jack Nervous Virus (English abstract only)

Abstract: The effects of some chemical disinfectants, organic solvents, hydrogen ions, heat, ultraviolet (UV) irradiation and ozone on the inactivation of striped jack nervous necrosis virus (SJNNV) were investigated. SJNNV was inactivated by contact with a final concentration of 50 ppm of sodium hypochlorite, calcium hypochlorite, benzalkonium chloride and iodine for 10 min at 20EC. Cresol concentrations of more than 10,000 ppm were required to inactivate SJNNV, and no inactivation of SJNNV by formalin was detected at any concentration tested. The effective concentrations of ethanol and methanol were 60% and 50%, respectively, but SJNNV was resistant to ether and chloroform. SJNNV was inactivated by high alkalinity, pH 12 for 10 min at 20EC, and also inactivated by heat treatment at 60EC for 30 min. Inactivation of SJNNV by UV irradiation was observed at an intensity of 410 ΦWcm^{-2} for 244 sec. Ozone at 0.1 mg/L as a total residual oxidant was required to inactivate SJNNV for 2.5 min. Washing fertilized eggs and the treatment of sea water with ozone decreased the rate of occurrence of SJNNV.

2.3.8 Bullock et al. (1997) – Rainbow Trout

Abstract: Ozone was added to water in a recirculating rainbow trout (*Oncorhynchus mykiss*) culture system just before it entered the culture tanks in an attempt to reduce the numbers of heterotrophic bacteria in system water and on trout gills, and to prevent bacterial gill disease (BGD) in newly stocked fingerlings. During four 8-week trials, ozone was added to the system at a rate of 0.025 or 0.036-0.039 kg ozone/kg feed fed. In the control, where no ozone was added, and in previously published research, BGD outbreaks occurred within two weeks of stocking, and these outbreaks generally required three to four chemotherapeutant treatments to prevent high mortality. In three of four trials where ozone was added to the system, BGD outbreaks were prevented without chemical treatments, but the causative bacterium, *Flavobacterium branchiophilum*, still colonized gill tissue. The one ozone test in which BGD outbreaks required two chemical treatments coincided with a malfunction of the ozone generator. Although ozonation did reduce BGD mortality, it failed in all trials to produce more than a one $\log_{10}$ reduction in numbers of heterotrophic bacteria in the system water or on gill tissue. Failure of the ozone to lower numbers of heterotrophic bacteria or to prevent the causative BGD bacterium from occurring on gills was attributed to the short exposure time to ozone residual (35 s contact chamber) and rapid loss of oxidation caused by levels of total suspended solids. Rationale for ozone's success at preventing BGD mortalities are not fully understood but may in part be due to improved water quality. Use of the lower ozone dosing rate (0.025 kg ozone/kg feed) appeared to provide the same benefits as the higher dosing rate (0.036-0.039 kg ozone/kg

feed fed); however, the lower ozone dosing rate was less likely to produce a toxic ozone residual in the culture tank and would also reduce ozone equipment capital and operating costs.

The overall objectives of this research were to demonstrate what effect ozonation would have when added at levels that were obtained by creating 3-4% ozone within the existing oxygen feed gas before it is transferred into the system. It is significant that the ozone is generated and transferred within an oxygen feed gas that was already required to provide a dissolved oxygen supersaturation within each culture tank's influent.

The objectives of the research reported here were to demonstrate the effects of ozone addition on outbreaks of BGD and on total heterotrophic bacteria concentrations.

Materials and Methods

Recirculating System: The recirculating system consisted of one fluidized-sand biofilter, two multi-stage low-head oxygenator, two microscreen filters, one cascade aeration column, and two cross-flow fish culture tanks. The system recirculated water in two parallel flow paths (a path for fish culture and a path for biofiltration and carbon dioxide stripping) connected within a common sump. In the fish culture path, approximately 720 L/min were split into two parallel streams that were first pumped through a LHO unit, were carried by gravity through the cross-flow fish culture tank, and were finally passed through the Triangle filter unit with 80 Φm opening sieve panels before dropping back into the sump.

Each cross-flow tank had a culture volume of 9.0 m^3, which was replaced 2.3 times per hour or about 55 times per day. In the biofiltration and carbon dioxide stripping path, approximately 760 L/min were pumped through a fluidized-sand biofilter, and then were cascaded counter-current to air within the carbon dioxide stripping column before returning to the sump. Partitions were placed within the common sump to reduce mixing between the fish culture path and the biofiltration/stripping path. The sump design allowed for the independent operation of the fish culture and biofiltration flow paths, which was particularly important during chemical treatment of the fish culture tanks.

Ozone Tests: Ozonation of the recirculating system was studied during four 8-week tests and an 8-week no ozone control. During the first two ozone trials, ozone was added only to the flow passing through the LHO unit preceding tank. Adding ozone prior to only one of the two culture tanks allowed study of whether dosing location impacted system performance. Ozone was added to both LHO units during the third and fourth ozone tests, which allowed maximizing the amount of ozone that could be added to this recirculating system without making additional structural modifications or without increasing oxygen usage beyond that required by the fish. Approximately 0.025 and 0.036-0.039 kg ozone were added per kg feed fed in the first two ozone tests and the last two ozone tests, respectively. Ozone addition was relatively constant during each test, except in trial 2 when the ozone generator failed.

Generated ozone was moved through stainless steel pipes to either one or both LHO units and was transferred to the recirculating flow just prior to entry into the culture tank. Adding ozone to the LHO within this configuration resulted in an ozone contact time within the water of only 35 s before it entered the cross-flow culture tank. Because cross-flow culture tanks are characterized

as completely mixed vessels, the ozone that entered the culture tank was immediately diluted to the concentration leaving the culture tank. Therefore, the culture tank provided additional time for ozone reaction and destruction.

Dissolved ozone was measured three times a week at the water inflow immediately after ozonation and within culture tanks. As an added safety measure oxidation/reduction potential (ORP) based control systems were used to prevent ozone residual from accumulating to toxic levels within the culture tanks.

Enumeration of heterotrophic bacteria and *flavobacterium branchiophilum*: The effect of ozone on the numbers of heterotrophic bacteria in the recirculating water and on rainbow trout gills and the presence of *F. branchiophilum* on gill tissue was determined as follows: The day before the fish were stocked, five were randomly selected, euthanized in tricaine methanosulfonate, and gill tissue was aseptically removed. A gill smear was prepared to detect *F. branchiophilum* by the indirect fluorescent antibody test (IFAT). Each stained smear was examined under oil immersion, using a fluorescence microscope with epi-illumination, and the number of clumps (three or more cells) of *F. branchiophilum* was counted in 50 microscope fields. For enumeration of heterotrophic bacteria, 0.48-0.52 g of gill tissue was aseptically weighed into a sterile 15-mm X 75-mm tube. Cold. sterile, pH 7.2 phosphate buffered saline (PBS) was added to prepare a 1:10 dilution. Each sample then was sonicated to remove bacteria, and serial $\log_{10}$ dilutions were prepared. Using the drop plate technique, six 50-ΦL drops each of selected dilutions were placed onto a 15-mm X 100-mm culture plate of plate count agar (PCA; Difco). Plate cultures were incubated at 25EC for 72 h, colonies were counted in each dilution, multiplied by the appropriate dilution factor, and reported as colony forming units (CFU) per gram of gill tissue. In the recirculating culture system water, samples were taken just prior to and immediately after the points of ozone addition (i.e., one or both LHO units) and from water within the culture tanks. Ten-fold dilutions were prepared using PBS; plate counts were performed as previously described, and bacteria reported as CFU/mL) of water.

For each of the four ozone tests and the no ozone control, gill and water samples were taken on day 7, 10, 14, 17, 24, 28, 35, 42, and 49 post stocking. In ozone tests one and two and the no ozone control, five fish from each train were examined each sample day for heterotrophic counts and IFAT examination. In ozone tests three and four, five fish were sampled for heterotrophic counts but, because of limited supply of antiserum, only three fish per tank were examined by IFAT.

Results: The addition of ozone in the four tests did not prevent colonization of *F. branchiophilum* on the gills or completely prevent mortality from BGD (Table 2.3.15). Additionally, ozone did not appear to reduce the numbers of heterotrophic bacteria on gill tissue or in the water by more than 1 $\log_{10}$ (Table 2.3.15). Ozone reduced water color and the concentration of nitrite, and oxidized the total suspended solids improving their removal across the Triangle microscreen filters. In the culture tanks, water pH ranged from 7.1-7.3, oxygen from 9.1-12 mg/L, total ammonia nitrogen from 1.1-1.3 mg/L, TSS from 2.9 to 6.3 mg/L, nitrite from 0.024-0.265 mg/L and temperature from 14.3-16.3EC.

Bacterial Gill Disease: *F. branchiophilum* was not detected on gill tissue before fish were stocked. Once fish were stocked, *F. branchiophilum* was detected on gill tissue within 10 days in

all trials (Table 2.3.15). The control and test two had a slightly higher percentage of *F. branchiophilum* positive fish. Some mortality from BGD occurred in all trials; but it was slightly higher in ozone trial two (when the ozone generator malfunctioned) and during the no ozone control. The higher percentage of fish carrying the bacterium and the necessity of chemical treatment in test two coincided with a 40% reduction in ozone production due to fouled dielectrics in the corona discharge cell of the ozone generator. In the control, four chemical treatments were required in each culture tank to prevent increased mortalities. However, multiple chemical treatments were not required to control mortality from BGD in three of the four tests in which ozone was added to the system (Table 2.3.15). In tests one, three, and four, mortality from BGD was self-limiting, and no treatments were required.

Heterotrophic Bacteria: Heterotrophic bacterial counts in C-1 and C-2 tank water during the control trial contained 3.1×10^4 bacteria/mL water, while gill samples from fish in the two culture tanks contained $3.9\text{-}5.8 \times 10^5$ bacteria/g tissue. Counts during the 8-week period for tests one and two showed a slight reduction of bacteria in culture tank water. The range in C-1 water was 4.5×10^3 to 6.8×10^3 CFU/mL; the sample site was directly before the point of ozone addition. The range in C-2 water was 1.4×10^4 to 1.8×10^5 CFU/mL; water in this tank should not have received any direct exposure to residual ozone in tests one and two. In tests three and four, when both tanks received ozone. counts ranged from 3.1×10^3 to 4.8×10^3 CFU/mL (Table 2.3.15).

There was no apparent effect of ozone on numbers of heterotrophic bacteria on gill tissue during the trials, counts ranged from 2.9×10^5 to 4.2×10^6 CFU/g tissue (Table 2.3.15).

Discussion: Prior to ozonation, BGD was a constant problem among newly stocked fish. During an 11-month period previous to ozonation, five groups of rainbow trout were stocked, and up to 30% of each group died because of BGD or a secondary amoebic infection despite regular chemotherapeutic treatments. In the ozonation study, BGD-associated mortalities also occurred on a regular basis when ozone was not added or insufficient ozone was added. Adding ozone appeared to lower total mortality and the number of clumps of BGD bacteria on gill tissue in tests one, three and four, compared to that in the control and test two, when the ozone generator failed. A total of 14 treatments was required to reduce BGD mortality in the two tanks in the control and test two, while no treatments were needed in the other trials. After ozone addition, only 1.7-4.1% of stocked fish died because of BGD, and chemical treatments rarely were required (Table 2.3.15). The benefits of adding ozone to this system were an overall improvement in water quality entering the culture tanks and, more importantly, a reduction of mortality due to BGD and a reduction in the need for chemotherapeutic treatments.

Table 2.3.15. Effect of' ozone addition on occurrence of' bacterial gill disease, fish mortality, and water quality parameters (Bullock et al., 1997)

Parameter	Control (no ozone)	Ozone Trial 1	2	3	4
BGD-induced mortalities, %					
Tanks C-1 + C-2	4.3	4.1	10.1	3.3	1.7
Treatments to control BGD [a], #					
Tank C-1	4	0	2	0	0
Tank C-2	4	0	4	0	0
Presence of *F. branchiophilum* on gills, percent positive (#/#) [b]					
Tank C-1	54 (27/50)	8 (4/50)	54 (27/50)	39 (9/23)	40 (12/30)
Tank C-2	44 (22/50)	24 (13/50)	46 (23/50)	57 (12/21)	30 (9/30)
Average number of clumps of *F. brachiophilum* per 50 fields on infected trout gills					
Tank C-1	4.0	0.45	8.0	5.0	5.0
Tank C-2	3.0	1.8	8.0	4.0	4.0
Heterotrophic bacteria on gills, CFU/g tissue x 10^4 ∀ s.e.					
Tank C-1	58.3 ∀ 8.4	37.9 ∀ 12.4	425 ∀ 166	425 ∀ 229	167 ∀ 111
Tank C-2	39.5 ∀ 7.4	29.2 ∀ 8.7	223 ∀ 49	205 ∀ 88	129 ∀ 75
Heterotrophic bacteria in water, CFU/mL x 10^3 ∀ s.e.					
Tank C-1	30.8 ∀ 10.3	6.8 ∀ 3.2	4.5 ∀ 1.1	4.8 ∀ 1.5	3.6 ∀ 2.2
Tank C-2	30.6 ∀ 8.2	18.0 ∀ 2.2	13.8 ∀ 2.0	3.1 ∀ 1.3	3.8 ∀ 2.5
Ozone concentration, Φg/L ∀ s.e.					
Tank C-1 infl	0	50.0 ∀ 12.9	180.0 ∀ 29.6	33.6 ∀ 15.6	87.3 ∀ 29.0
Tank C-2 infl	0	0	0	18.2 ∀ 7.5	65.5 ∀ 22.0
Tank C-1	0	10.0 ∀ 6.4	24.5 ∀ 1.6	5.5 ∀ 3.9	11.8 ∀8.0
Tank C-2	0	0	0	3.6 ∀ 3.6	10.9 ∀ 6.7
Ozone-induced mortalities, %					
Tanks C1 + C2	0	0	0	3.9	5.0
Temperature, EC ∀ s.e.	15.2 ∀0.2	14.3 ∀ 0.1	15.6 ∀ 0.1	16.3 ∀ 0.1	15.2 ∀ 0.1

a 12 mg/L chloramine-T for 1 h or 2 mg/L Roccal for 1 h.
b Number of trout positive/number of trout examined.

An indirect measure of residual ozone is the water's oxidation reduction potential (ORP) which is a measure of a water's potential to oxidize and is thus a measure of the water's potential to disinfect or to kill fish. ORP can be monitored and used to control ozone addition to ensure that the desired treatment objective has been achieved and to ensure that ozone residual is not in the fish culture tank. A safe ORP for freshwater appears to be between 300-350 mV, depending upon pH.

2.3.9 Abad et al. (1997) – Mussels Depuration

Abstract: Studies were conducted in the common mussel (Mytilus spp.) to evaluate the public health implications derived from shellfish contamination with human pathogenic enteric viruses. In bioaccumulation experiments, it was verified that after 6 h of immersion of mussels in marine water contaminated with high levels of clay-associated enteric adenovirus (type 40) and human rotavirus (type 3), between 4 to 56% of the seeded viruses were adsorbed onto shellfish tissues,

mainly in the gills an digestive tract. We investigated the occurrence of wild-type enteric viruses in mussels from sites with different levels of fecal pollution. Pathogenic viruses could be detected in mussels from areas that, following current standards based on bacteriological quality, should be regarded as unpolluted, safe for swimming, and suitable for harvesting shellfish. Cooking experiments performed with contaminated mussels revealed that 5 min after the opening of the mussel valves, rotaviruses and hepatitis A virus still could be recovered in steamed shellfish. Under commercial deputation conditions, health-significant enteric viruses, such as rotavirus and hepatitis A virus, could be recovered from bivalves after 96 h of immersion in a continuous flow of ozonated marine water. Routine screening of bivalves for the presence of health-significant enteric viruses before public consumption may help in the prevention of outbreaks among shellfish consumers.

Materials and Methods

Experimental Virus Contamination of Mussels: Five groups of 40 mussels (*Mytilus spp.*) were contaminated over a 24-h period with approximately 10^7 to 10^8 most probable numbers of cytopathogenic units (MPNCU) of clay-associated human rotavirus Ito ^{r}P13 (HRV), human enteric adenovirus type 40 (ADV), the cytopathogenic HM-175 strain of hepatitis A virus (HAV) and poliovirus 1, strain LSc 2ab (PV), in 4-liter tanks of estuarine water (salinity 3.2%, conductivity <3,500 mmhos, temperature 21 to 23EC). Mussels were starved for 24 h before each experiment.

Virus Inactivation in Mussels by Cooking: Mussels were experimentally contaminated as described above with HAV, PV, and HRV. The mussels were steamed and samples taken at the opening of the valves and 5 min afterwards. Survival of the viruses in the cooked mussels were determined by calculating the log (Nt/No), where No is the titer of the virus at time zero and Nt is the titer at various assay time periods.

Mussel Depuration: Deputation was performed over 96 h by placing groups of 40 mussels that had been experimentally contaminated with HAV PV, HRV, and ADV in a continuous flow of ozonated marine water in 50-liter tanks. The mussels were assayed for infectious viruses after 20, 48, and 96 h of depuration.

Environmental Sampling: Mussels and seawater samples were collected from coastal areas showing three different levels of fecal pollution. According to bacteriological standards determined in previous studies and unpublished data by the authors, the sampled shellfish beds may be qualified as heavily polluted, polluted, and unpolluted. Samples were kept at 4EC storage while in transit to the laboratory, where they were processed within 24 h of collection.

Bacteriological Analysis: Fecal coliform and fecal streptococci counts were determined in mussel and seawater samples according to Standard Methods for the Examination of Water and Wastewater. Mussel meat was homogenized in distilled water (1:7, wt/vol) prior to the bacteriological assays.

V**irus Assays:** Viruses were extracted from the mussels and concentrated from the resulting eluate by polyethylene glycol precipitation. In the bioaccumulation experiments, whole mussel meat and carefully dissected tissues (gills, digestive tract, and mantle lobes) were assayed at the

designated time intervals for viruses. Only whole mussel meat was processed in the deputation experiments. PV and HAV were propagated and assayed in BGM and FRhK-4 cells, respectively. HRV and ADV were cultivated and assayed in MA-104 and CaCo-2 cell monolayers, respectively. Viral enumerations in mussel tissue or water samples were performed by calculating the MPNCU per g or mL, respectively, by infecting cell monolayers grown in 96-well microtiter plates. Eight wells were infected for each dilution, and 20 ΦL of inoculum were added to each well. The analysis of variance (ANOVA) test was used to determine significant differences between the behaviors of viral strains.

Wild-type virus determinations were carried out on 100 g (wet weight) of mussel tissue stomached in 700 mL of distilled water. Viral concentrates (4 to 12 mL) were kept at -80EC until assayed. Wild-type enteroviruses were enumerated by plaque formation by inoculating confluent BGM cell monolayers. HRV were assayed by an indirect immunofluorescence test infecting MA-104 cell monolayers. The presence of wild-type HAV was evaluated by molecular hybridization with a ^{32}P-labeled cDNA probe of the complete HAV genome. Positive signals were detected by autoradiography. All experiments were conducted at least in duplicate, and all virus assays were performed twice.

Results

Bioaccumulation of Viruses in Mussels: After feeding mussels with high levels of clay-associated ADV and HRV, infectious viruses were readily detected in mussels after 1 h of contact time. Maximum titers of viruses adsorbed to mussel meat were observed after 6 h: 1.1 x 10^4 MPNCU/g for HRV and 1.7 x 10^5 MPNCU/g for ADV. Infectious virus titers declined thereafter. The water holding the mussels was monitored for the presence of infectious viruses throughout the experiment. ADV figures per liter of water were 2.5 x 10^7, 1.7 x 10^7, and 4.0 x 10^6 MPNCU at times 0, 6, and 24 h, respectively. At the same sampling times, HRV levels in water were 8.0 x 10^6, 5.0 x 10^6 , and 8.0 x 10^5 MPNCU/ liter. ADV and HRV adsorbed to mussel tissues after 6 h represented 25 and 35% of the total seeded viruses, respectively. In another set of experiments, 56% of hepatitis A virus and 4% of poliovirus were adsorbed to mussels after 6 h.

Tissue Distribution of Viruses in Mussels: Dissected tissues and intervalvar fluid from experimentally contaminated mussels were assayed for infectious HRV and ADV. For HRV, the highest virus numbers were found in the gills and labial palps, followed by the digestive tract, intervalvar fluid, and mantle lobes. The highest levels of ADV were detected in the intervalvar fluid, followed by the gills, digestive tract, and mantle lobes. After 6 h, the percent tissue distribution of detected adsorbed rotavirus was 48% in gills, 26% in digestive tract, 4% in mantle lobes, and 22% in intervalvar fluid. For ADV, after the same contact time, these figures were 31 % in gills, 8% in the mantle lobes, and 19% in the digestive tract, while 42% was detected in the intervalvar fluid.

Virus Inactivation in Mussels by Cooking: The virus inactivation curves are depicted in Figure 2.3.12. At the opening of the valves, all three assayed virus strains could be recovered from steamed mussels. Five minutes after the opening of the valves, PV was no longer detectable in cooked mussels. However, HAV and HRV could be detected, showing a reduction in the original titer below 3 log units.

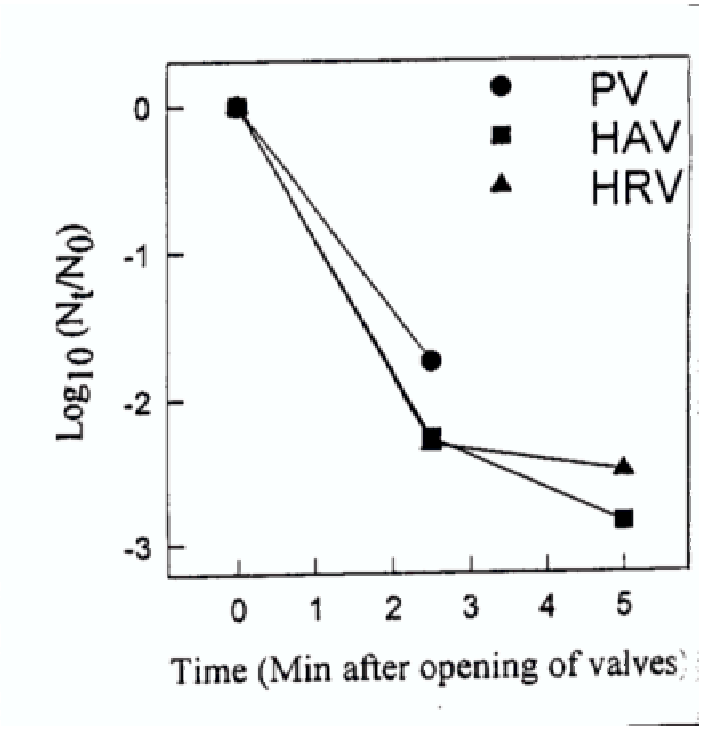

Figure 2.3.12. Inactivation of viruses in shellfish by cooking. PV poliovirus; HRV human rotavirus; HAV hepatitis A virus (Abad et al., 1997.

Removal of Viruses by Depuration: The effects of depuration on the removal of enteric viruses from mussel tissue are shown in Figure 2.3.13. PV, which appeared to be the most susceptible virus to depuration, showed a 3-log-titer reduction (LTR) after 48 h, and became undetectable thereafter. After 96 h, ADV showed a 99.82% reduction, while the reductions in infectivity for HAV and HRV were 98.71% and 96.99%, respectively.

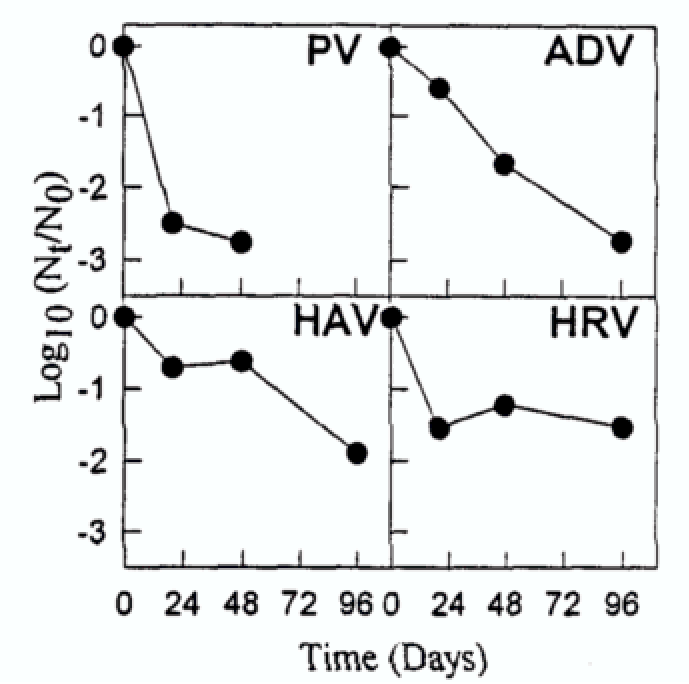

Figure 2.3.13. Removal of viruses in shellfish by depuration. PV poliovirus; ADV enteric adenovirus; HRV human rotavirus; HAV hepatitis A virus (Abad et al., 1997).

Discussion: In the present study performed with mussels contaminated with PV, HRV, and HAV, between 0.41 % and 1.78% of the initial viruses still could be detected in steamed mussels at the time when valves are opened. Five minutes later, 0. 32% and 0. 14% of the initial infectious HRV and HAV, respectively, were recovered, while PV was no longer detectable. Under commercial depuration conditions (using ozone) HRV, HAV, and ADV persisted much longer than PV. In these conditions, shellfish are kept in a flow of clean seawater to allow them to purge themselves of their contaminant load. No correlation was observed between bacterial indicator microorganisms and viruses.

2.3.10 Kötters et al. (1997) – Redfish Aboard Fishing Vessels

Abstract: Studies were conducted aboard fishing vessels and in laboratories to assess effects of transport or holding in ozonated water on bacteriological quality and shelf life of redfish (rockfish, *Sebastes spp.*). Ozone appeared to promote detachment of the surf ace slime of the fish; some of the surface bacterial film is removed together with the surface slime. Intermittent ozonation of the water during transport reduced bacterial count and improved shelf life of redfish by approx. 36 h. Simulation trials in the laboratory gave different results: bacterial counts were higher on fish held in ozonated water than on control fish held in non-ozonated water. It is suggested that this difference may be attributable to lower initial freshness of redfish used in the laboratory study. In spite of the higher bacterial count, trimethylamine-N concentrations were lower in fish held in ozonated water than in fish held in non-ozonated water; this may indicate that ozonated water had a selective action on the microflora.

Introduction: A number of fishing vessels are using ozone as a disinfectant to conserve catch quality until it is unloaded at processing facilities. Ozone is a rapid oxidant widely used as a

disinfectant for drinking water. In drinking water treatment, however, the concentration of organic compounds is very low relative to that found in the hold water of a fishing vessel transporting its catch. This study therefore examined the effectiveness of ozonation for controlling bacteria from the time the fish were caught until unloading at the processing plant. This paper compares microbial counts from treated and untreated samples taken onboard a commercial fishing vessel during transport to a processing facility. An attempt also was made to simulate the field experiment in the laboratory.

Material and Methods:

On Board the Commercial Fishing Vessel 'Arctic Ocean': The 'Arctic Ocean' is typical of vessels which ozonate their catch in the local fishery. It is a mid-size fishing vessel with an overall length of 74.6 ft (678.9 m). The fish tank sampled had a capacity of 30,000 lbs (13,636 kg) of fish (excluding water) and a total volume of about 20 m^3 sea water. Freshwater ice was loaded prior to departure.

Bacteriological samples were taken from the time of landing the catch through transportation until off-loading and processing. Ozonated and control fish were sampled to determine growth of surface bacteria. Weather conditions prevented a more frequent sampling of the bacterial flora during transport to the processing plant. Sampling was initiated during the cruise from October 26 to November 6, 1992, inclusively.

The average time spent at sea by a fresh-fish trawler usually is no longer than 10 days. In the present study, fish stored on board had a maximum 'age' after catching of about 8 days. To maintain the freshness of the catch, fish in the storage tanks were cooled with flaked ice. The storage tank water was recirculated and ozonated up to three times a day, depending on the length of time the fish were held in storage.

Investigations During Unloading at a Fish Processing Plant: Microbial samples were taken from ozonized fish on their way from the boat to the filleting line, as well as from the surrounding water in the hold. Figure 2.3.14 shows the principle steps in a typical groundfish fishery. Roman numerals refer to the samples described in the text. Fish are vacuum-pumped ashore and transferred via a conveyor belt (metal screen or plastic) to the indoor cache tank for short-term storage in ice water (temperatures near 0EC).

Microbiology: 'Psychrotrophic' bacteria are a major contributor to the spoilage of seafood. The incubation temperature of the plates was kept #21EC. Incubation was conducted on board the 'Arctic Ocean.' The medium used was always non-selective. Tryptic Soy Agar (TSA) and the drop plate method used (ICMSF 1978). Serial dilutions were made in 0.1% peptone broth.

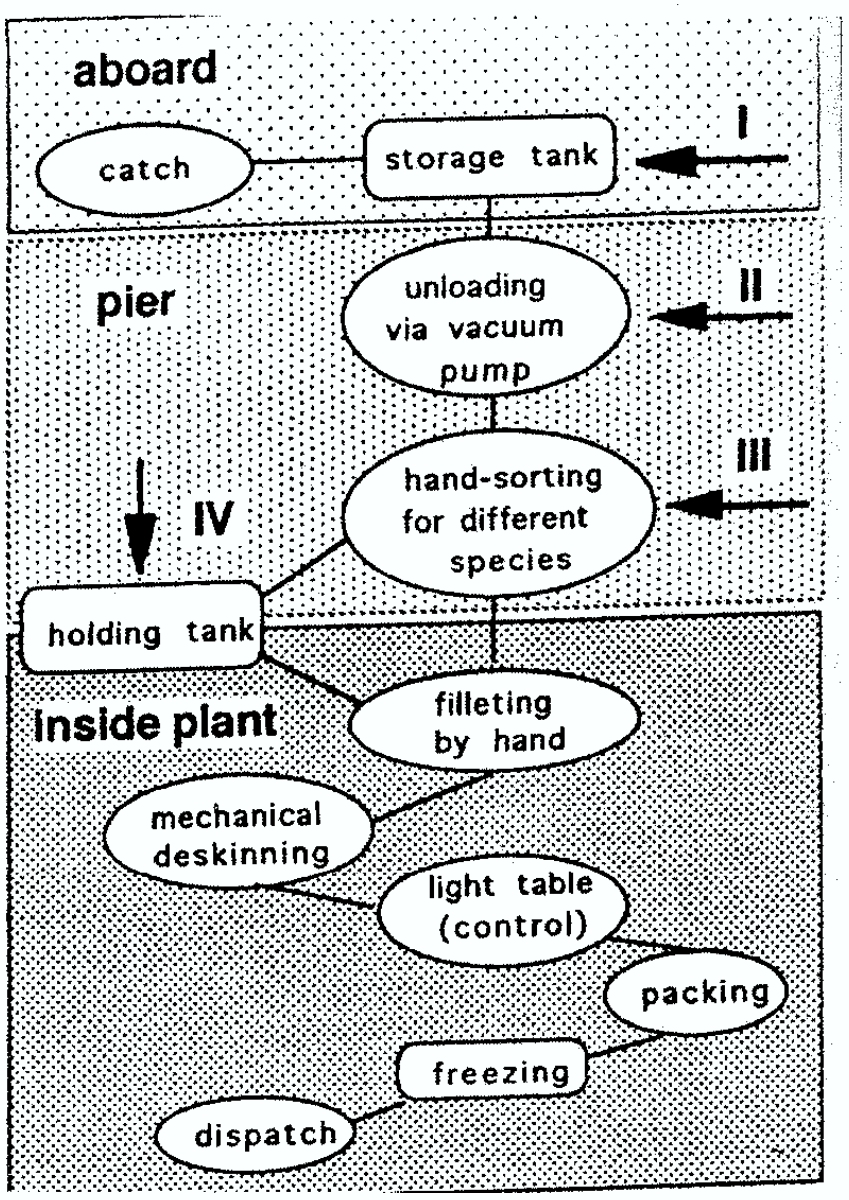

Figure 2.3.14. Flow chart of fish processing in a typical plant. Major steps of processing for fresh fish from catch to dispatch are indicated. Each step follows its own time course. Fish may be stored for various time periods after being pumped ashore. Catch usually is cooled in ice-water near 0EC. Arrows and Roman numerals indicate sampling points of this study (I = storage tank aboard the fishing vessel; II = after the vacuum pump; III = conveyor belt during hand sorting; IV = holding (cache) tank (Koetters et al., 1996). The effect of added ozonated water on the psychrotrophic bacterial flora in iced storage tanks was studied by frequent sampling from the fish skin at intervals between catch and landing (unloading of catch at processor's pier). Stored fish treated with ozone were compared with an untreated control Kötters et al., 1997).

Water Samples: Three water samples were taken from each of the sample points I, II and III. Each 50 mL was kept on ice in a sterile plastic bag for less than 7 h. 20 ΦL of each sample was serially diluted to 10^{-6}. Of each dilution, 20 ΦL was pipetted onto TSA plates in duplicate using the drop plate method.

Sampling from Fish Surfaces: A sterile aluminum foil template (16 cm^2 onboard~ 50 cm^2 at the processing plant) was placed on the fish surface; the area inside the template was swabbed with a sterile cotton swab first dipped in 0.1% solution of peptone water. The swab then was washed carefully in 5 mL of the same peptone solution. A 20 ΦL sample was serially diluted to 10^{-3}. Twenty ΦL of each dilution was pipetted onto TSA media in duplicate.

Samples were taken at points I to IV (see Figure 2.3.14) and plates prepared at the processing plant where they were incubated at 21EC and counted after 24 and 48 h.

On board the 'Arctic Ocean' the plates were counted after 72 h, at which time almost constant temperature conditions prevailed. Incubation of samples took place in a storage room near the bow of the ship. The temperature curve of the incubator was recorded (∀ 2EC) via a mechanical temperature recorder. Average incubation temperature during the cruise was 10EC.

The first catch (24 October 1992) was sampled immediately after hauling the net and after 2, 4, 6, and 8 days of storage. For comparison and control, 12 fish from the same catch were packed at the time of catch in four polyethylene bags (3 fish per bag) and stored in the same tank as the ozonated fish. Three of the control fish (1 bag) were sampled at each of the sampling times, parallel to the ozonated fish. Fish may be stored for various time periods just after pumping ashore. When stored in water, the catch is cooled by ice water at temperatures near 0EC.

After passing through the vacuum pump, fish were transported via conveyor belts (plastic surface or metal meshes). From there, further conveyor belts distributed the fish over various distances to the processing lines where fish were filleted by hand.

Simulation Experiment: After chemical disinfection with 10,000 ppm sodium hypochlorite, each of the stainless steel tanks (capacity each 800 L) were filled with 200kg 'rockfish' (*Sebastes brevispinis*) in the round. Fish were caught about 5 days prior to the start of the experiment. Fish were not treated with ozone during storage in the fishing boat. Fish were shipped on ice from the processing plant to the laboratory at Univ. of British Columbia. The fish were layered into each tank whereby each layer of fish was covered with flaked ice, with another layer of fish placed on top of the ice layer until the tank was about 80% full. The tanks then were filled with cold tap water.

Figure 2.3.15 depicts the layout of the system used to simulate on-board ozonation of fish. During the simulation trial both tanks were treated twice per day. When tank O was treated, the O_2 flowed through the system but the ozone generator was turned off and tank T was disconnected from the system. When ozone was applied, tank T was treated while tank O was disconnected from the system. The three-way valves (V) were used to switch the flow to the oxygenated tank. Treatment periods lasted for 20 min using the setup shown in Figure 2.3.15. Both ozonated and oxygenated samples were treated in alternate cycles. Excess water

originating from melting ice was removed daily and replaced by an adequate amount of ice (about 20 kg) in order to maintain a constant temperature in the storage tanks.

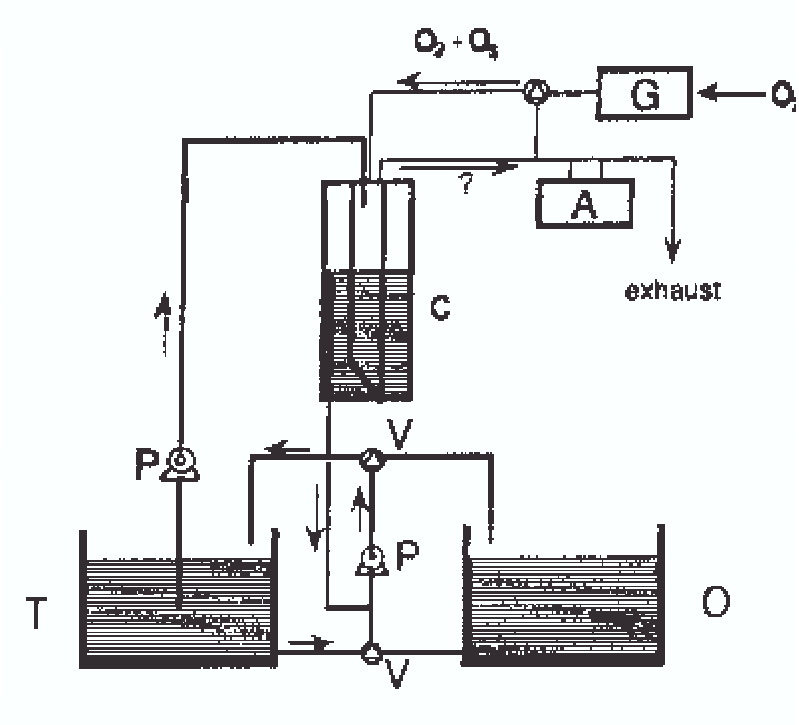

Figure 2.3.15. Schematic diagram of the experimental unit for simulating storage of fish on board a fishing vessel; G: ozone generator; A: ozone analyzer; C: Contacting column; P: Pump; V: 3-way valve: T: ozonated tank; O: oxygenated control; arrows indicate direction of flow (Kötters et al., 1996)

The lines and the bubble column which were isolated from the tanks after each circulation/ozone run were cleaned and sanitized with 10,000 ppm of sodium hypochlorite.

Three fish were removed from each tank after selected circulation runs (in the morning of day 0, 1, 3, 5, 7 and 9) for microbial and color evaluation. The remains of each fish after removal of samples for microbiological and color evaluation were kept at -30EC in a sealed polyethylene bag for further chemical and sensory evaluation. Microbial samples were taken from the exposed skin near the pectoral fin as well as from the covered gill epithelia.

The contacting chamber for producing a standardized level of ozone in the simulation experiment consisted of two concentric cylinders constructed from 1.25-cm thick Plexiglas. The inner cylinder was equipped with a spray bar to distribute the water in an ozone-oxygen atmosphere with a known concentration of total radical oxidants. The water has to pass to the submerged bottom part of the cylinder, while most of the fine gas bubbles merge into larger ones which out-gas into the larger cylinder opposite to the drain pipe, thereby minimizing the carry-over of gas bubbles into the outlet.

Ozone Generation: Total Radical Oxidants (TROs, usually called 'ozone') were produced in an electrical discharge (Sander ozone generator Model 201), operating at 7 kV and using high purity oxygen (dried to <3 ppm moisture). The output of the unit was regulated by varying the flow-through rate of the gas and calibrated according to standard methods of TRO determination.

A corona discharge unit (Azcozone) was used on board the trawler 'Arctic Ocean' with its output characteristics depending on the frequency of the generator on-board the vessel (110 VAC; air flow oxygen-enriched; dried, operating at 10 kV). No measurements were taken on the performance of the unit.

Ozone Determination: Under field conditions, residual ozone was determined with the DPD method, using a test kit and a portable spectrophotometer. The accuracy for ozone measurements when compared to the Indigo method (Bader and Hoigné 1979) was $\forall$ 0.1 mg L^{-1}. In the simulation experiment, ozone (total radical oxidants) was determined in the gaseous phase. Determinations were carried out by directly measuring the absorption at 254 nm with a type G ozone analyzer (Sander, Germany). The best observed transfer efficiency was 44% at maximum water flow due to high turbulence. This high flow was used to guarantee a constant total radical oxidant level in the treatment unit.

Decomposition of ozone in water depends on temperature. pH and substrate type and concentration. Organic load greatly influences the half-life of total radical oxidants. In order to estimate the decomposition rate of ozone in storage tank water, a series of measurements was taken in: distilled sterile water, tap water, 'cache water', 0.1 % peptone solution, and 0.1 % tryptic soy broth.

Results:

Ozonation and Bacterial Load of Catch: As can be seen in Figure 2.3.16, the effect of ozonated water on the total bacterial count of skin samples from fish in the storage tank was a 90% reduction of initial counts compared to non-ozonized fish samples. Values varied between 0.7 and 1.5 log less than the counts on the untreated fish.

Except for the initial retardation of bacterial activity, bacterial load on fish skin increased in stored samples, and therefore the difference between controls and fish exposed to ozonation remained almost identical from day two onwards. The largest difference in bacterial counts was observed on day two. This likely was due to rapid initial growth of bacteria on freshly caught non-ozonated fish. Thereafter, the growth of the bacterial populations seem to slow considerably for several days, while the ozonized fish in storage followed the same trend at a somewhat lower total level. Thereafter the rate of bacterial increase was the same in both ozonated and control samples.

Microbial Changes During Unloading at the Pier: As can be seen in Figure 2.3.17, in spite of mechanical and manual handling, surface bacteria counts decreased when fish were transported from the holding tanks on-board via vacuum pumps to the pier. Simultaneously, the number of bacteria increased in the pumped water.

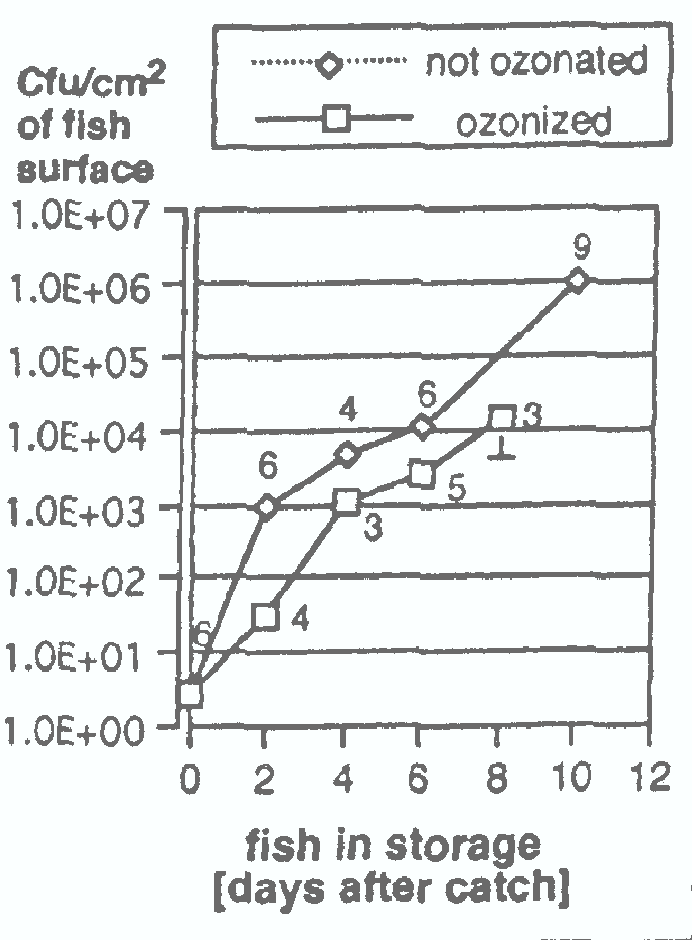

Figure 2.3.16. Comparison of psychrotrophic microbial growth on ozonized and control fish between capture and unloading at the processing plant wharf. Numbers = sample size, bars = standard deviation (Kötters et al., 1996).

Simulation Experiment: In order to verify the results obtained in the field studies and to further study the relevance of observed reduction of bacterial counts when pumping ozonated fish ashore, the storage of the catch on board was simulated experimentally in the laboratory. The results of the simulation experiment followed a different trend from those observed on the fishing vessel (see Figure 2.3.18).

During the first three days of storage, no difference in total psychrotrophic bacterial counts was observed. Thereafter, the colony forming units (CFU) on both surfaces (skin and gills) were slightly lower (0.5 unit on the log scale) on the oxygenated fish than on the ozonated fish. The difference between both data sets related to skin-associated bacteria tended to be smaller near the end of the observation period.

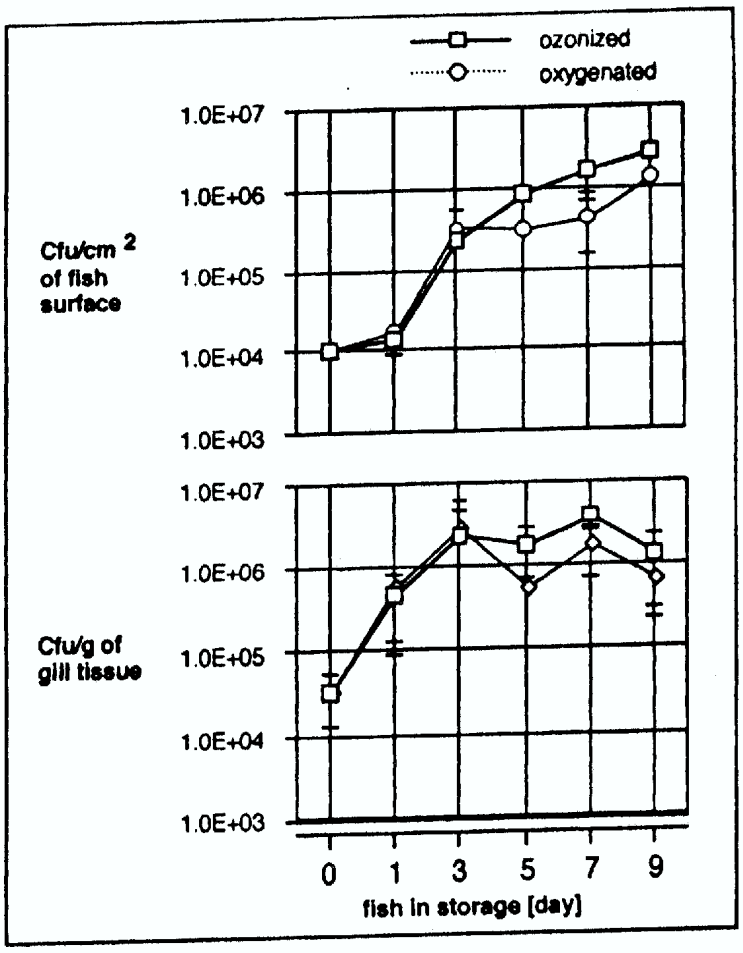

Figure 2.3.17. Microbiological changes of ozonized fish landed. Microbial counts from surface samples of fish indicated the number of microorganisms attached on the fish surface. Counts decreased during handling (pumping) of fish from the boat to the processing plant (Kötters et al., 1996).

The same fish were tested for TMA-N. After the fifth day this important quality-determining parameter showed a rapid increase, indicating strong growth in the activity of TMAO-reducing organisms.

Discussion:

In this study we identified two mechanisms by which ozonation affects the bacterial flora of fresh fish. The first mechanism involves direct inactivation of bacteria. This effect, especially in water, is well-known and often reported. In relation to drinking water, however, the tank water in which fish are stored contains much higher levels of dissolved organic matter. Due to the non-specific nature of ozone reactions, ozone attacks organic molecules as well as microorganisms. The ozone demand of the dissolved organics makes it difficult to maintain higher ozone concentrations over the time period between treatments. From the data in Figure 2.3.19 it is obvious that the actual concentration of radical oxidants in the field trials declined rapidly and that the intervals between treatment (12-24 h on-board) certainly were too long to allow any

residual ozone to remain in the storage tanks on-board the trawlers between ozonation treatments. The maximum observed residual ozone (total oxidative radical) concentration determined on the fishing boat was 0.1 ppm.

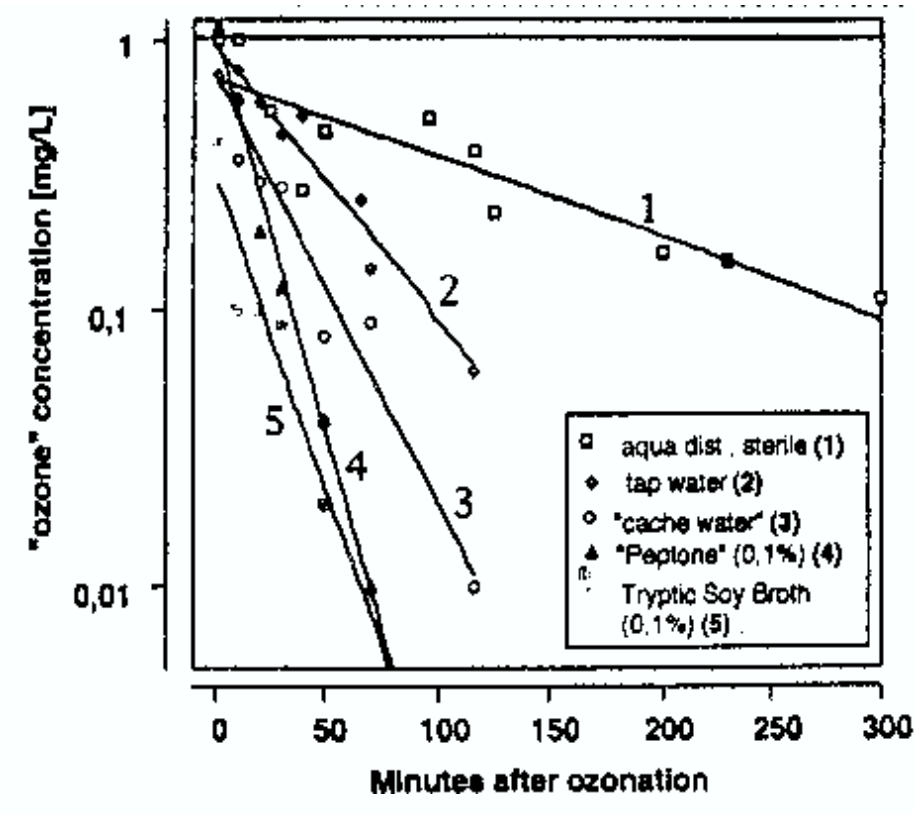

Figure 2.3.18. Simulated medium-scale storage offish in ozonized (30 min per day) and oxygenated (control) water. Effects on colony forming units (CFU) in gill tissue and skin surface samples. Number of determinations per sample point = 3; bars = standard deviation (Kötters et al., 1996).

Though the dissolved organics in the tank water prevented attainment of concentrations of total oxidative radicals as high as 0.4 ppm, counts on ozonated fish on-board the boat were lower than those of the control fish. This observation suggests a possible second ozone-related mechanism controlling the development of the bacterial community. Ozonation appears to ease the separation of slime and its associated bacterial flora from the fish.

The polysaccharide slime serves as the initial growth medium for the microflora while fish are in storage. While the bacteria attack the outer surface of slime, these bacteria also are susceptible to oxidative radicals in the tank water. If the bacteria can penetrate the slime the innermost areas of the slime may, however, provide some bacteria with a refuge where they may proliferate, safe from the immediate effects of the ozone. The ultimate effect of this structure of slime, bacteria and oxidative radicals is that the slime on ozonated fish appears to be more easily removed from the body of the fish by the hydraulic shear forces associated with pumping the fish off the boat. With the removal of the slime the number of bacteria on the surface of the fish decreases (Figure 2.3.16).

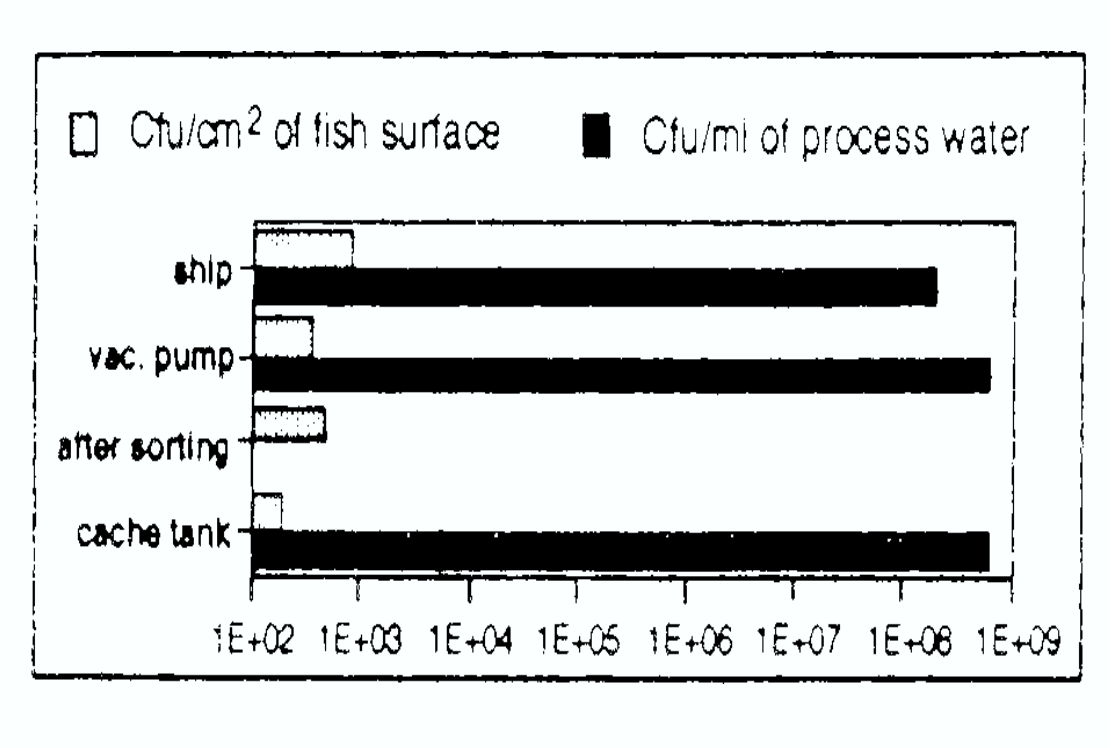

Figure 2.3.19. "'Ozone' (total radical oxidant) decomposition in different standard media (semi-logarithmic) at 20EC. 1=distilled water; 2=tap water (chlorinated). The data referring to media containing different organics are labeled "3-5". These are: 3=water from a fish processing plant holding tank; 4-Peptone 0.1%; 5=Tryptic soy broth 0.1%. Media were ozonated for 5 min in an attempt to reach an initial concentration of 1 mg L^{-1}. Due to the high organic content of media 4 and 5, their immediate reactivity with ozone did not permit reaching this intended initial concentration (Kötters et al., 1997).

From the bacterial count data obtained after pumping fish ashore, it also can be assumed that bacterial mats which had developed on the surface slime of fish while in storage on-board were washed down together with the slime. It is likely that the slime was freshly secreted by the fish during catch (a typical stress response) and that ozonation on-board immediately after catching seems to precondition this slime to allow easy separation of the top slime layer from the fish surface. This separation mechanism seems to be beneficial and therefore desirable, preventing elevated bacteria levels entering the processing plant together with freshly-landed fish.

The precise mechanism leading to this effect is not entirely clear. However, two scenarios for the derivation of this effect suggest themselves. The bacteria utilizing the interior of the slime layer may weaken the adherence of the slime to the fish. When exposed to hydraulic shear forces the slime then may separate from the fish's body. Alternately, residual oxidants in the medium may change the structure of the polysaccharide-containing slime matrix, changing its physical properties, thereby allowing it to be more easily removed by the force of moving water. The former scheme should leave a richer bacterial fauna on the skin after removal of the slime. The data in Figure 2.3.16 demonstrates that the opposite is the case. Lower surface bacteria

counts are measured after removal of the slime. This implies that some chemical change occurred in the polysaccharide slime which aided its removal from the fish skin.

On board the fishing vessel there was a retardation of microbial growth by about 36 h (on average). This may be interpreted as an equally long extension of maintenance of the quality of fresh fish. In the preceding paragraphs which discuss changes in the removal of the polysaccharide slime, it becomes clear that bacteria near the fish's skin, normally protected by the slime cover, would have higher exposure to the total oxidative radicals in the water when the initial ozonation removed the top slime layer.

The high organic load in the chill-water probably prevented the ozone from reacting with pigments in the skin and gills. The change in aroma between the ozonated and control tank indicated that ozone had an effect on volatile compounds associated with fish spoilage in the present study.

In the simulation experiment, TMA-N levels showed some quality advantage in storage in the ozone-treated fish when compared with oxygenated fish. despite the higher total bacterial counts. This observation suggests an important shift within the bacterial populations towards spoilage-causing bacteria in the oxygenated fish samples. This is not at all detectable when simply looking at the total colony counts. In spite of the higher total counts. ozone treatment seems to have had a selective disinfecting quality. especially. with regard to TMAO-reducing organisms. However, 5 days of storage seems to be the limit for controlling the growth and activity of TMAO-reducing organisms. A sudden and remarkable increase of TMA-N occurred in all samples from day 6 onwards. Ozonation seems only to delay the proliferation of the volatile components, as TMA-N was only about 33% (day 7) and 28% (day 9) less in ozonated than in oxygenated samples. Our observations lead to the conclusion that the use of ozonated ice water aboard fresh fish trawlers should be started immediately after the catch to lower the initial bacterial counts. These bacteria certainly form the base for later growth and determine the shape of the growth curve. Continued intermittent treatment of fish with ozone while in storage, starting immediately after the catch, seemed effective as a means to extend shelf-life. The present study suggests that intervals between treatments might be shortened to improve the results. However, further studies are required to verify this suggestion.

2.3.11 Mimura et al. (1998a) – Japanese Flounder (in Japanese with English abstract)

Abstract: Delayed hatching of eggs (DHE) of Japanese flounder, *Paralichthys olivaceus* was observed, following rinsing with seawater containing ozone-produced oxidants (OPO). Although they did not hatch at the same time as normal eggs, the embryos continued to develop and remained alive for two more days. When the embryos were exposed to 0. 8 mg O_3/L for 10 min, DHE occurred 91.3-95.1%, less than 3 min, exposure did not affect hatching. When the embryos were exposed to 3-10 mg O_3/L for one min, DHE occurred 94.3-98.1%. At concentrations of less than 2 mg O_3/L, however, very little effect of OPO on hatching was observed. When the embryos at blastula to heart beat stage were exposed to OPO 0. 8 mg O_3/L for 6 min, delayed hatching of eggs was observed at almost the same rate.

2.3.12 Mimura et al. (1998b) – Japanese Flounder and Ozone-Produced Oxidants in Seawater (in Japanese with English abstract)

Abstract: We tried to quantitate residual oxidants by measuring trihalogenated methanes generated from reacting resorcin with TRO (total residual oxidants). Our results showed that the residual oxidants in OPO (ozone-produced oxidants) seawater, TRC (total residual chlorine) seawater and electrolyzed seawater were mostly hypobromous acid. When seawater containing hydrogen peroxide or povidone-iodine was treated with resorcin, no trihalogenated methane was detected. In addition, when NaClO was added to seawater diluted 3 - 30 times with distilled water, both chlorine-containing and bromine-containing oxidants were detected.

Delayed hatching was observed frequently in Japanese flounder, *Paralichthys olivaceus*, eggs exposed to OPO seawater, TRC seawater or electrolyzed seawater. Occurrence of the delayed hatching increased rapidly when the oxidant concentration increased to 2 mg O_3/L or higher. No increment of dead eggs was observed. When the eggs were exposed to seawater containing hydrogen peroxide or povidone-iodine, few delayed hatching of eggs were found. Most of the eggs that failed to hatch died.

Petitioners' Note: This article is included to point out that whether ozone or chlorine is used to treat seawater, bromide ion is oxidized to produce hypobromous acid (HOBr), a brominating agent capable of producing brominated trihalomethanes (and, presumably) other brominated organic compounds). Use of chlorine, however, also forms mixed bromo-chloro-trihalomethanes (and, presumably, other mixed bromo-chloro-organics). These types of halogenated organics as well as the HOBr itself might be responsible for the observed effects, rather than the chlorine or ozone added initially to seawater. The reactions of chlorine and ozone with bromide ion to produce HOBr is seawater are very rapid (half-lives of ozone or chlorine in seawater measured is seconds).

2.3.13 Goché and Cox (1999) – Chum Salmon

This study was designed to test the impact of ozone on fresh Chum Salmon while at the same time attempting to address several specific questions and concerns as follows:

1. Effects of ozone at different levels.

2. Effect when ozone is used at final wash stage only.

3. Effect when used at both pre-wash and final wash stages.

4. Document if OSHA/NIOSH safety levels are reached in the atmosphere at proposed levels. (Note: OSHA/NIOSH levels are 0.1 ppm for an 8 hour time weighted average, or 0.3 ppm for 15 minutes).

5. Determine if ozone masks odors on product or through inhibition of olfactory senses at different levels of decomposition.

6. If masking occurs, determine at what level and rate at which senses/odor returns.

7. Comparison of impact on microbial load at different levels of treatment versus untreated controls and chlorinated controls.

8. Effect on ability to prolong maintenance of quality/slow decomposition through sensory trials.

9. Determine if ozone use causes or accelerates development of oxidation and rancidity.

Fresh Alaska Chum Salmon, *Oncorhynchus keta*, were offloaded and obtained from a processing plant in Ketchikan, Alaska. Fish were of uniform quality and were handled in the same manner by plant and team personnel. Twelve whole/round untreated and unprocessed fish were selected as control samples. Another 12 fish of uniform size were selected and processed under normal conditions with chlorine-treated (0.5 ppm free chlorine) city water, also for control samples.

Testing was conducted over a 2-day period in 2 phases. Phase I (1 application - final wash), and Phase II (2 applications -- pre-wash and final wash). On both days the fish were dressed, processed and sprayed with ozone treated wash water of low level (0.5 ppm residual), medium level (1.0 ppm residual) and high levels (1.5 ppm residual), 12 fish at each level. The chlorine was stripped from the wash water during this procedure. Masking tests were performed to determine whether ozone created an inhibitory effect in detecting odors of decomposition from either atmospheric build-up or from direct contact with fish flesh.

The fish were shipped, with portable temperature recorders, to Seattle, WA and sent to an independent microbiological testing laboratory. The Total Plate Counts of the ozone treated samples were compared to those of both unprocessed control samples and those processed under normal, chlorinated water, conditions. Additionally, the rate of quality deterioration was tracked for all samples to determine if there were any differences between ozone-treated samples at various levels and chlorinated or untreated samples.

Summary of Results

1. It appears from this study that changes in equipment design would be necessary in keeping with the unique conditions in each plant and further to minimize the ozone off-gassing effects.

2. Masking does not appear to be an issue either in terms of inhibition of olfactory senses except at levels far higher than those permitted by established safety limits.

3. Masking of odor-producing fish flesh from direct contact is not an issue when product is evaluated before treatment or at least 10 seconds after treatment.

4. TPC counts are greatly reduced in comparison to untreated whole round samples by both chlorine and ozone. However, there appears to be no advantage (greater efficacy) to levels above 0.5 ppm residual, or to more than one application.

5. Quality deterioration was slowed by a single application of ozone (final wash) at all residual levels applied in this study in comparison to all other treatment types.

6. Acceleration of oxidation in fresh fish by the use of ozone is not a significant factor as the fish spoils prior to any such development.

Ozone Equipment, Measuring Devices, and Set Up: A basket washer was converted to an ozone sprayer for the purpose of this test. Spray nozzles on the top and bottom of the unit were utilized to provide for uniform coverage on both sides of the fish. These nozzles consisted of 6 top and 6 bottom TG 3.5 standard cone, unijet nozzles with a capacity of 0.58 gpm at 30 psi and 0.67 gpm at 40 psi. Nozzles were approximately 13 inches apart from top to bottom, with the top row approximately 8 inches above the fish and the bottom approximately 2.5 inches below the fish. An ozone destruct unit was mounted on top of the basket washer, along with plastic curtains at the entry and exit points, in order to draw out atmospheric ozone within the chamber and prevent its escape through the openings. Chlorine was stripped from the water in the process line as well as from the water feeding the ozone unit by two charcoal filters. The ozone was generated via corona discharge. The feed gas to the ozone generator was supplied using an oxygen concentrator. The ozone was introduced into the water using a contactor which incorporates an injector.

For Phase I, Day 1, fish were butchered by the crew in their standard fashion and then placed two at a time on a lattice-style plastic tray. This tray then was pushed into the washer for a timed interval of 10 seconds. Fish were individually bagged after treatment to prevent mingling of samples and direct contact with each other which would skew the results of the bacteriological and sensory testing. Throughout, fish were handled with gloves and touched only around the head and tail regions.

Phase II, Day 2 involved the same procedure with the addition of a spray application (pre-wash) prior to butchering. Swabbing of both sides of the fish carried out in Seattle (TPC testing) was confined to the mid-region of each fish.

Ozone Application Procedures: On August 31, 1999 Chum Salmon, *Oncorhynchus keta*, were offloaded from a tender at a seafood plant in Ketchikan, Alaska. All fish selected were of good freshness and quality as evidenced by firm texture, clear eyes, and bright red gills with sea-fresh odor. The fish were selected from the offload grading table at around 6:30 AM and were iced immediately.

Control Samples:

1. Select 12 fish of uniform quality from the iced tote.

2. Package and label for shipping, 6 fish per carton along with gel ice.

At 12:00 PM 12 whole/round untreated fish were randomly selected to be used as control samples. They were individually bagged in plastic sleeves and packed 6 fish per wet lock shipping carton with 6 gel-ice packs per case (3 on the top and 3 on the bottom). A portable temperature recorder was placed in one of the cases of the whole/round fish cartons in order to track any temperature abuse during shipment. The cases were placed in refrigerated storage until shipment later that day.

At 12:25 PM, 12 more fish were randomly selected from the same iced tote to be used as control samples. The samples were dressed and treated normally with chlorinated city water (0.5 ppm free chlorine level). The fish then were bagged, packed, shipped and handled the same way as the whole/round salmon. Because the whole/round and the chlorinated control samples would be shipped together, only one temperature recorder in the whole/round control samples was necessary.

Phase I – Outline:

I. Process samples at 3 residual ozone levels (0.5 ppm, 1.0 ppm, and 1,5 ppm) -- final wash.

   A. Strip chlorine from process water

   B. Measure pH of water

   C. Start ozone generator and test for residual ozone in the spray

   D. Select 12 fish of uniform quality.

   E. Process fish with chlorine stripped from process water.

   F. Record atmospheric ozone levels continuously from start to finish.

   G. Treat fish with 0.5 pprn residual ozone, final wash (1 treatment)

   H. Package fish for shipment with Gel ice.

   I. Perform Masking Test.

   J. Place in refrigerated storage until shipping

II. Process 12 fish samples at increased residual ozone level 1.0 ppm -- final wash -- same as above.

III. Process 12 fish samples at increased residual ozone level 1.5 ppm -- final wash -- same as above.

0.5 ppm Residual O_3 Application: Starting at 2:47 PM, the ozone spray unit was set at 0.5 ppm residual ozone. The gas phase ozone concentration ranged from 31.4 g/m^3 to 32.2 g/m^3. The water flow rate ranged from 7.30 to 7.58 gpm.. The oxygen gas flow rate to the ozone generator was 2.5 liters/minute oxygen at 10 psi/g. The inlet pressure to the injector was measured at 86 psig and the outlet pressure was 33 to 34 psig. The factory room temperature was noted to be 60.1EF, with a relative humidity of 64.5-75.9%.

At 2:58 PM, the ozone residual stabilized at 0.5 ppm and treatment began. The fish were placed on a lattice style plastic tray 2 fish at a time, pushed into the spray unit and timed at a 10 second dwell. Upon removal, fish were immediately bagged individually in plastic sleeves, and packed

in cartons 6 fish per box in the same manner previously described. All fish were finished and packed at 3:08 PM.

1.0 ppm Residual O_3 Application: At 3:55 PM, the ozone spray was increased to an application level 1.0 ppm residual ozone. The gas phase ozone concentration ranged from 27.7 g/m^3 to 29.1 g/m^3. The water flow ranged from 7.30 to 7.58 gpm. The pH of the water was recorded as 7.59. The oxygen gas flow rate to the ozone generator was 3.2 liters/minute oxygen at 10 psig. The inlet pressure to the injector was measured at 85 to 86 psig and the outlet pressure was 33 to 35 psig. The factory room temperature was noted to be 60.8EF.

All 12 fish were exposed to the 1.0 ppm residual ozone application and were completely packaged by 4:12 PM. The fish were sprayed, handled and packaged the same way as the other fish samples and were immediately placed in refrigerated storage.

1.5 ppm Residual O_3 Application: By 5:08 PM an ozone spray application level of 1.5 ppm residual ozone was reached and maintained. The gas phase ozone concentration ranged from 30.2 g/m^3 to 31.0 g/m^3. The water flow rate was 7.30 gpm. The oxygen gas flow rate to the ozone generator was 3.8 liters/minute oxygen at 10 psig. The inlet pressure to the injector was measured at 86 psig and the outlet pressure was 36 psig. The factory room temperature was noted to be 60.1EF with a relative humidity of 63.1%. All 12 fish were exposed to the 1.5 ppm residual ozone application and were completely packaged by 5:12 PM. The fish were handled and packaged the same way as the other fish samples and were immediately placed in refrigerated storage.

The whole/round control samples, the chlorinated control samples, the 0.5 ppm ozone applied samples, and the 1.0 pprn ozone applied samples were put on a plane that same night and shipped to Seattle. During the night the wetlocks were maintained in refrigerated storage until they were picked up the next morning and delivered to an independent testing laboratory TPC testing according to AOAC-approved methods.

The 1.5 ppm ozone applied samples did not ship on Tuesday due to time restrictions for airport delivery. (Those samples remained in refrigerated storage at the plant and were shipped with fish from the following day of testing).

Phase II – Outline:

I. Process samples at 3 residual ozone levels (1.5 ppm, 1.0 ppm, and 0.5 ppm) -- Prewash and Final wash.

   A. Strip chlorine from process water

   B. Start ozone generator and test for residual ozone in the spray

   C. Select 12 fish of uniform quality.

   D. Wash 12 fish with 1.5 pprn residual ozone level prior to butchering

   E. Process fish with chlorine stripped from process water.

F. Record atmospheric ozone levels continuously from start to finish.

G. Treat fish with 1.5 ppm residual ozone level, final wash

H. Package fish for shipment with Gel ice.

I. Perform Masking Test

J. Place in refrigerated storage until shipping

II. Process 12 fish samples at residual ozone level 1.0 ppm -- Pre wash and final wash – same as above.

III. Process 12 fish samples at residual ozone level 0. 5 ppm -- Prewash and final wash – same as above.

Wednesday, September 1, 1999, 6:30 AM; Chum Salmon are offloaded from a tender vessel at the dock of the processing plant. About 60 fish of uniform quality were selected from the sorting belt and immediately placed in ice.

1.5 ppm Residual O_3 Application: By 1:35 PM, an ozone spray application level of 1.5 ppm residual ozone was maintained. The gas phase ozone concentration was 32.2 g/m^3. The water flow rate ranged from 7.02 gpm to 7.58 gpm. The oxygen gas flow rate to the ozone generator was 3.8 liters/minute oxygen at 10 psig. The inlet pressure to the injector measured at 85 to 90 psig and the outlet pressure was between 33 and 37 psig. The ambient air temperature was recorded as 59.2EF with a relative humidity of 81.8%.

Starting at 1:43 PM, 12 fish were selected from the iced tote. They were first put through a 10-second ozone applied spray in the whole/round form. The fish then were dressed (headed and gutted). Chlorine was stripped from the process water while cutting and cleaning the fish. The H&G fish then were put through another 10-second ozone spray. They then were bagged individually, packed 6 fish per carton, Gel pack inserted, labeled and placed in refrigerated storage.

1.0 ppm Residual O_3 Application: At 2:08 PM, the ozone spray had been decreased to an application level of 1.0 ppm, residual ozone. The gas phase ozone concentration ranged from 30.0 g/m^3 to 30.9 g/m^3. The water flow rate was 7.02 gpm. The oxygen gas flow rate to the ozone generator was 3.2 liters/minute oxygen at 10 psig. The inlet pressure to the injector was measured at 85 to 86 psig and the outlet pressure was 32 to 33 psig.

At 2:08 PM, 12 fish again were chosen at random from the iced tote and directly run through the ozone spray for 10-seconds. They were then dressed (H&G), using chlorine stripped water. All fish were passed through the ozone spray one more time for 10-seconds. The fish were packaged in the same manner as before.

0.5 ppm Residual O_3 Application: At 2:35 PM, the ozone spray unit was monitored and stabilized at 0.5 ppm residual ozone. Water pressure ranged from 40.7 g/m^3 to 40.8 g/m^3. The water flow rate was recorded at 7.30 gpm. The oxygen gas flow rate to the ozone generator was

2.5 liters/minutes oxygen at 10 psig. The inlet pressure to the injector was measured at 86 psig and the outlet pressure was 33 psig.

At 2:38 PM, the spray maintained a residual ozone application level of 0.5 ppm. At that time, l2 fish were run through the spray in the round form. The fish were kept under the ozone-applying spray for 10 seconds and then taken out the other end of the unit and were immediately headed and gutted. The fish then were passed through the spray for a second wash under the ozone spray. The fish were packaged in the same manner as the previous samples. All fish were packed, in refrigerated storage and ready for shipment at 2:47 PM.

The two cartons of Phase I 1.5 ppm and all the cartons of fish from Phase II were picked up from the processing plant and delivered to the airport for shipment. The cartons arrived in Seattle later that night and were placed in refrigerated storage until morning.

Inhibitory Impact from Direct Contact of Ozone with Fish Flesh: On Day 1, a fish with off odors at Class III was split and both sides passed through the ozone application unit at a 10 second dwell time = residual ozone treatment level 1.0 ppm. Immediately out of the sprayer the odor was completely masked on both of the sides. After 5 seconds the odor began to return slightly. After 10 seconds the full odor again was present.

On Day 2, the steps described above again were performed with a fish of the same quality = residual ozone treatment level 1.5 ppm. Results were identical.

Shipment Receipt and Handling: Samples were shipped in wetlocks containing 6 fish each and 6 Gel packs. On September 1, Phase I samples (single treatment = final wash) were picked up at the airport, taken for swabbing, and transported to the Surefish laboratory in Seattle, Washington for sensory evaluation. The Phase I samples received on September 1 consisted of:

2 cartons of Whole/Round Controls
2 Cartons of Cl_2 Controls
2 Cartons of PH I (Phase I) Ozone Treated Samples at 0.5 ppm
2 Cartons of PH I Ozone Treated Samples at 1.0 ppm
Total Samples - 48 fish

Two cartons of PH I samples treated at residual ozone level 1.5 ppm were left in refrigerated storage in Ketchikan to ship with the following day's samples as they were not completed in time for the flight schedule. On September 2, the remaining samples consisting mostly of Phase II product (two applications pre-wash and final wash) were transported for swabbing and taken to the Surefish laboratory in Seattle for analysis. These samples consisted of:

2 Cartons of PH I Ozone Treated Samples at 1.5 ppm residual
2 Cartons of PH U Ozone Treated Samples at 0,5 ppm residual
2 Cartons of PH H Ozone Treated Samples at 1.0 ppm residual
2 Cartons of PH 11 Ozone Treated Samples at 1.5 ppm residual
Total Samples - 48 fish

Temperature Data: Temperature recorders were placed inside numerous cartons at random. Temperature data represents the point at which the sample box was closed to the time in which

each was first opened for swabbing. Samples were shipped from Ketchikan on the same day of preparation with the exception of the 1.5 ppm treatment level for Phase I, which had to be shipped later. For both shipments, product then sat overnight in refrigerated storage at the airport until they were picked up.

Microbiological Testing:

Testing Procedure: Product was tested for Total Plate Count (TPC). Fish were handled in a sanitary fashion and AOAC-approved methods were followed. Cartons were brought in by Surefish personnel from the company truck one at a time. Surefish staff handled the fish by the tail while microbiological testing lab staff performed the swabbing. Fish were removed from their protective sleeves, the outside of each fish was swabbed, and each was returned to their original sleeve and container. Swabbing was carried out by utilizing a sterile sponge and swabbing the side of each fish. This occurred first on one side (10 cm x 10 cm area), then on the other (10 cm x 10 cm area) with the same sponge. Total area swabbed per fish = 200 cm^2. Each fish was swabbed in the mid region below the dorsal fin (so that the dorsal fin served as a reference point for consistency). The belly region and proximal areas were avoided. Each sponge was placed in a sterile sample bag. Dilutions were performed at 1 to 100 and 1 to 1000 using 100 mL of sterile buffered dilution water. Diluents were plated onto Standard Methods agar and incubated at 35EC for 48 hours. Visible colonies then were counted and the total microbial load per sample calculated. The raw data in Tables 2.3.16 (Day 1 samples), 2.3.17 (Day 2 samples), and Figure 2.3.20 reflect the TPC counts per 200 cm^2 for each sample.

Table 2.3.16. Raw data. Day 1 samples swabbed on Sept. 1. Single application of ozone at three different levels in ppm (Goché and Cox, 1999)

Sample	TPC per 200 cm^2	TPC sorted	Mean	Mean & STDEV with outliers removed
1	37000 WR #1	13000	17350	15564; STDEV: 5839
2	18000	16000		
3	26000	18000		
4	13000	24000		
5	24000	26000		
6	16000	**37000**		
				(**Group1)**
7	14000 WR #2	6200		
8	11000	10000		
9	6200	11000		
10	15000	14000		
11	18000	15000		
12	10000	18000		
37	1900 CL #1 H&G	1900	2400	2400 (no outliers) STDEV: 962
38	3400	1900		
39	1900	2500		
40	2500	2800		
41	2800	3100		
42	3100	3400		
				(Group 2)
43	1600 CL #2 H&G	200		
44	3600	1600		
45	3100	1800		
46	200	2900		
47	1800	3100		
48	2900	3600		
25	1400 PH I, 0.5 #1	200	2125	1664, STDEV: 1007
26	3300	1400		
27	1800	1700		
28	1700	1800		
29	2200	2200		
30	200	3300		
				(Group 3)
31	200 PH I, 0.5 #2	200		
32	800	800		
33	1400	1400		
34	2500	2500		
35	2800	2800		
36	7200	**7200**		

Sample	TPC per 200 cm^2	TPC sorted	Mean	Mean & STDEV with outliers removed
13	2400 PH I, 1.0 #1	1300	3625	2864, STDEV: 1244
14	2300	1500		
15	5600	2300		
16	3600	2400		
17	1300	3600		
18	1500	5600		
				(Group 4)
19	3700 PH I, 1.0 #2	1700		
20	12000	2800		
21	1700	3000		
22	2800	3600		
23	3600	3700		
24	3000	**12000**		

- PH I = Phase I; WR = Whole/Round control samples; CL = chlorinated H&G control samples.
- Sample numbers are out of sequence due to random case selection at micro lab. Data then was regrouped in logical sequence from controls up to increasing treatment levels for the purpose of this analytical comparison.
- Bolded numbers denote outliers.

Table 2.3.17. Raw data. Day 2 samples swabbed on Sept. 2. Two application points of ozone at three different levels in ppm * (Goché and Cox, 1999)

Sample	TPC per 200 cm^2	TPC sorted	Mean	Mean & STDEV with outliers removed
1	5600 PH I, 1.5 #1	2900	10917	7555; STDEV: 8086
2	49000	3400		
3	3400	4200		
4	26000	5600		
5	4200	26000		
6	2900	**49000**		
				(**Group5**)
7	4800 PH I, 1.5 #2	1700		
8	3900	3300		
9	3300	3900		
10	21000	4800		
11	5200	5200		
12	1700	21000		
13	2800 PH II, 0.5 #1	2100	4775	4118 STDEV: 2032
14	2600	2200		
15	8400	2600		
16	5000	2800		
17	2200	5000		
18	2100	8400		
				(Group 6)
19	12000 PH II 0.5 #2	3000		
20	3000	3000		
21	6800	4600		
22	4800	4800		
23	4600	6800		
24	3000	**12000**		
25	1200 PH II, 1.0 #1	1200	2608	2191, STDEV: 1002
26	4200	1400		
27	2400	2200		
28	2200	2300		
29	2300	2400		
30	1400	4200		
				(Group 7)
31	7200 PH II, 1.0 #2	500		
32	1800	1800		
33	2100	2100		
34	3100	2900		
35	500	3100		
36	2900	**7200**		

Sample	TPC per 200 cm^2	TPC sorted	Mean	Mean & STDEV with outliers removed
37	28000 PH II, 1.5 #1	2400	16225	16225,(no outliers) STDEV: 17444
38	8800	8800		
39	2400	28000		
40	32000	32000		
41	44000	44000		
42	50000	50000		
				(Group 8)
43	9600 PH II, 1.5 #2	3000		
44	6400	3200		
45	3200	3400		
46	3900	3900		
47	3400	6400		
48	3000	9600		

- PH II = Phase II;
* Samples 1-12 were from Phase I, Day 1. Shipped out with second day's results as treatment of these fish was not completed in time to ship the same day.
S Bolded numbers denote outliers.

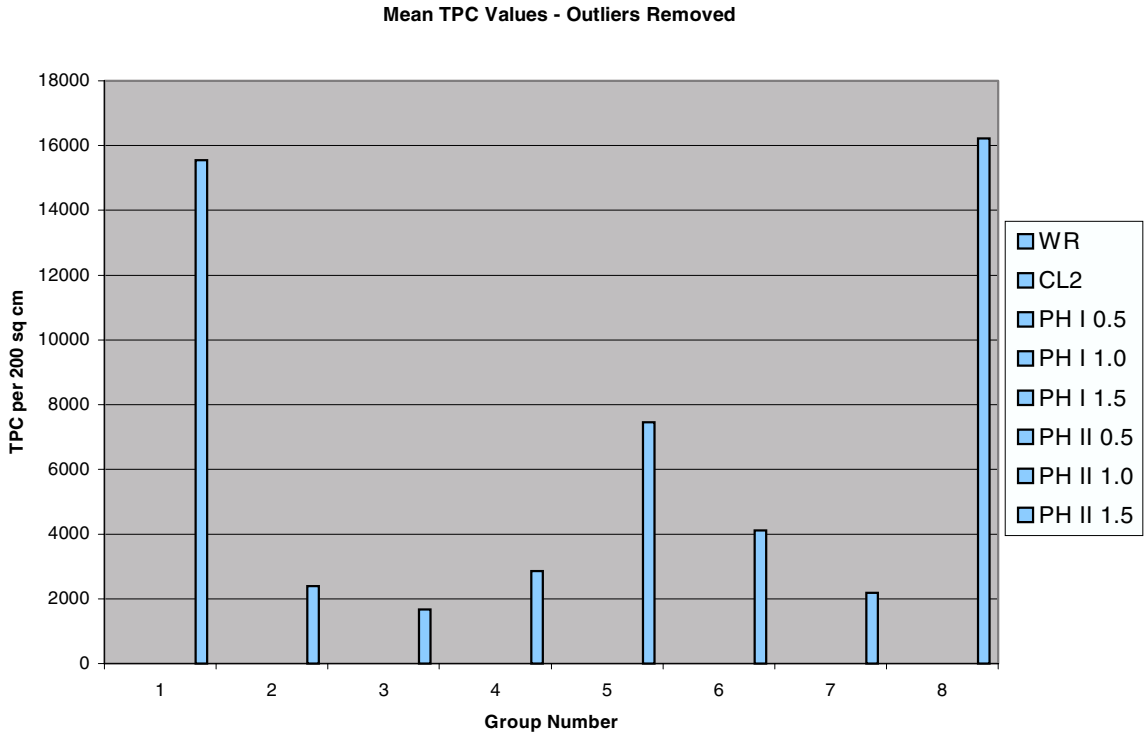

Figure 2.3.20. Mean TPC values – outliers removed (Goché and Cox (1999).

Conclusions – Microbiological Testing Results: Concerning the impact on TPC levels of increasing levels of ozone and 1 versus 2 application points, the data demonstrates that ozone is equally effective in comparison to chlorine. However, there was no increased kill factor at high ozone levels and/or at two treatment applications. Specifically:

1. Examination of the means with outliers removed for each group shows that for both phases (1 treatment and 2 treatments, respectively), the residual ozone application levels of 0.5 ppm and 1.0 ppm were just as effective as chlorine in reducing the levels of TPC.

2. Both ozone and chlorine had a substantial impact upon TPC in comparison to untreated control samples (whole/round samples) in all cases except PH II at 1.5 ppm.

3. There appears to be no advantage to applying residual ozone application levels beyond 1.0 ppm, or to more than one treatment step. There is no greater affect on TPC in comparison to whole/round controls beyond 1 treatment level at residuals of 0.5 and 1.0 ppm.

4. The residual application level of 1.5 ppm yielded curious results, particularly regarding PH II. The microbial load increased for PH I at the 1.5 ppm application level in comparison to other treated samples at PH I. However, the result is still significantly lower than the whole/round untreated controls, and therefore although the increase occurred it is not of particular significance. The same cannot be said for PH II at 1.5 ppm. This group (Group 8) had TPC levels that were essentially the same as the whole/round control samples, with much greater variation in the results between individual fish (i.e., a greater spread or larger standard deviation about the mean). This occurred in spite of the fact that these samples were treated twice (pre-wash and final wash) and at a higher level of ozone than the previous two groups on that day. This may have been the result of several factors which are unverifiable at this point and are purely speculation pointing toward potential areas of further study.

    A. The samples from Day 2 were similar in quality, collected, stored, and handled in the same way as Day 1 samples. Therefore whole/round untreated controls were not collected for comparison. This was an oversight as in retrospect this may have explained the TPC results for some of the fish in Phase II.

    B. Another issue may be the presence of psychrotrophic (able to grow at cold temperatures below 50EF) versus non-psychrotrophic bacteria. The intent of the study was to evaluate the overall impact upon total bacteria load. Perhaps the bacteria present on some of the samples from Day 2 contained a higher number of psychrotrophic bacteria. If this were true and the ozone had a greater impact upon the non-psychrotrophic microbes, the "cold tolerant" bacteria would be allowed to proliferate under refrigerated conditions, prior to swabbing, with little or no competition from other organisms.

    C. **Oxygenation:** Due to a higher microbial load present on the fish from PH II, and/or on the equipment, the ozone was "used up" after being applied to the samples prior to butchering. Were this the case, surviving bacteria may have been

"fed" by the additional oxygen once the ozone was depleted, allowing for more rapid growth which the second (final wash) treatment was insufficient to address.

D. **Fluctuations in Water Quality:** Variations in pH and organic materials can significantly impact effectiveness of ozone. Organic matter in the water can use up residual ozone in the same way as matter present on the fish before or after butchering and on the processing equipment.

E. **Possible Breakdown of Biofilm:** If microorganisms occur in clumps and these "clumps" are broken up during treatment, plate counts can increase exponentially. Thus elevations in TPC are not necessarily indicative of bacterial growth.

F. **"Ozone is highly effective against all microorganisms, but each class has its own rate of kill".** This is related to item "B" above, whereby it is also possible that some of the samples in PH II had different microorganisms present which require a greater treatment level or increased dwell time in comparison to those present in the other samples.

The above potential factors may account for the higher TPC levels in Phase II samples, but of course are purely speculation, as the exact cause is not known. These speculations provide areas for possible additional studies or for modifications when designing in-plant systems.

If we disregard Phase II for the moment and examine Phase I only at different levels of residual ozone, the following conclusions can be drawn:

1. As described earlier, the TPC levels for samples treated with chlorine and those treated at 0.5 ppm residual ozone are comparable.

2. At 1.0 ppm residual ozone, the results are higher but still comparable. At 1.5 ppm (shipped with Day 2 samples), the TPC levels are even higher. There appears to be no advantage to the 1.5 ppm application level even during Phase I.

All of the above points to the original conclusion. That is, based upon this study, increasing levels beyond 1.0 ppm residual ozone and applying more than one dose did not yield the expected further decrease in TPC levels. Further, these data also seem to indicate that, for the dwell times applied, the residual application level of 0.5 ppm ozone was just as effective as the 1.0 ppm level.

These conclusions are borne out through comparison of means and standard deviations. Further, a mulitvariant ANOVA was conducted by Christina DeWitt and Michael Morrissey (Oregon State University Seafood Laboratory) on the raw data utilizing StatGraphics Plus Software. The following conclusions are excerpted from their report "Surefish Statistical Report on ozone data" (after outliers have been eliminated):

1. At a confidence level of 95% ($p<0.05$) "there was a main effect... of both treatment and duplicate on Total Plate Counts (TPC). Replicates did not have a significant effect on the variability of TPC. Treatment with either chlorine or ozone at 0.5 and 1.0 ppm significantly reduced bacterial counts. Ozone application was just as effective as

chlorine. In addition, there was no significant difference between one or two applications of ozone ... two applications of ozone at 1.5 ppm and the control are now virtually identical in TPC. In addition, one application of ozone at 1.5 ppm is no different than the applications of chlorine or lower levels of ozone ... There is an overall trend, however, of increasing applications of ozone resulting in increasing bacteria".

Again with outliers removed, the data was transformed to $\log_{10}$ to isolate the effect of the treatments only (and minimizing the impact of variations in bacterial growth rate). The following conclusions apply as a result of an additional mulitvariant ANOVA:

2. At a confidence level of 99.995% ($p<0.005$), "again, application of ozone (0.5 and 1.0 ppm) is just as effective as treatment with chlorine. In addition, one application at 0.5 ppm appears to have a more significant effect on bacterial reduction than higher application levels of ozone."

Note: Although elevations in TPC occurred with some of the treated samples as described above, it is important to note that TPC is not an indicator of the presence of pathogenic microorganisms, and that in nearly all cases the TPC level was significantly reduced by exposure to ozone.

Sensory Analysis Results: The visual and organoleptic analysis employed to track deterioration rates at the Surefish Seattle Laboratory seemed to indicate rather clearly that the rate of deterioration of samples treated with ozone during Phase I was slowed compared to all other samples.

Oxidation/Rancidity Results: With respect to oxidation/rancidity development, very few samples showed signs of oxidation. Although 5 out of the 6 fish that did were treated with ozone, the discoloration occurred late in the trials. Therefore, fresh fish would spoil before oxidation/rancidity became a factor. Oxidation/rancidity would be more of a factor with frozen salmon. An earlier study conducted by the Fishery Industrial Technology Center ("Effectiveness of Ozone-Treated Wash Water and Ice on Keeping Quality and Stability of Sockeye Salmon" - J.S. Lee and D.E. Kramer, 1984 -- concluded that the "...expert panel did not find salmon stored in ozone-treated ice for 21 days, and subsequently stored frozen for six months, to be rancid").

Summary: The data generated during these trials indicate that ozone is at least as effective as chlorine as measured by the impact upon Total Plate Count. Further, sensory trials demonstrated a reduction in the deterioration rate of the fish treated with ozone, with no significant corresponding increase in oxidation/rancidity development. The potential for masking from ambient ozone levels or from direct contact with fish flesh was shown to be a non-issue when operations are carried out within the limits established by OSHA/NIOSH for ozone.

SUMMARY OF FISH SECTION

Substantial data collected in studies with shrimp, mussels, and several varieties of fish show antimicrobial efficacy of ozone to be equal or better than chlorine in most applications studied. Hatchery studies show benefits of reduced disease incidence, less mortality of hatchery stock, and shorter growth cycles with several species. Antimicrobial action of ozone has been shown against many species of bacteria, fungi, viruses, and cyst microorganisms. Absence of adverse sensory effects and freedom from harmful oxidation byproducts confirm the desirability of ozone use in processing fish products for human consumption.