DIRECT FOOD ADDITIVE PETITION

NAME OF PETITIONER: Dee M. Graham, Ph.D., The Electric Power Research Institute, Food Technology Alliance, Energy Delivery and Utilization

POST OFFICE ADDRESS: 2747 Hutchinson Court, Walnut Creek, CA 94598
Tel/Fax 925-938-0928
E-mail: deeg213050@aol.com or dgraham@epri.com

DATE: 2 August 2000

NAME OF FOOD ADDITIVE AND PROPOSED USE:

Ozone as an Antimicrobial Agent for the Treatment, Storage and Processing of Foods in Gas and Aqueous Phases

2 August 2000

Petitions Control Branch
Food and Drug Administration
Department of Health and Human Services
Washington, DC 20204
DEAR SIRS:

The undersigned, **The Electric Power Research Institute**, submits this petition pursuant to section 409(b)(1) of the Federal Food, Drug, and Cosmetic Act with respect to ***Ozone as an Antimicrobial Agent for the Treatment, Storage and Processing of Foods in Gas and Aqueous Phases***.

Attached hereto, in triplicate, and constituting a part of this petition, are the following:

A. The name and all pertinent information concerning the food additive (**ozone**).

B. The amount of the food additive (**ozone**) proposed for use and the purposes for which it is proposed, together with directions, recommendations and suggestions regarding its proposed use, including proposed levels and conditions of use to ensure safety of both the foods to be treated, food processing plant personnel, and the environment.

C. Data establishing that **ozone** will have the intended physical or other technical effect.

D. A description of practicable methods to determine the amount(s) of ozone in water or air that comes into contact with raw, processed or finished foods. Because of the very short half-life of ozone, especially when contacting materials capable of being oxidized (e.g., foodstuffs), no ozone is anticipated to be incorporated into the foods themselves. Therefore, the need to analyze the treated foodstuff(s) for residual ozone is not applicable.

E. Full reports of investigations made with respect to the safety of **ozone**.

F. Proposed tolerances for **ozone**.

G. This Food Additive Petition does ***not*** propose to modify an existing regulation pursuant to section 409(c)(1)(A) of the Act.

H. A claim for categorical exclusion under section 25.32(r) of 21 CFR Chapter 1 (4-1-98 Edition).

We respectfully request that FDA submit this petition to expedited review, since the FAP describes the use of ozone to reduce the levels of human pathogenic microorganisms (e.g., *E. coli, Salmonella, Campylobacter,* and in some cases, *Cyclospora* and *Listeria*) or their toxins in various foods. Also attached is a Resolution of Support for the broad application of ozone in food and agriculture as was described in the Expert Panel Declaration Of GRAS Status for Ozone in Food Processing on April 10, 1997 (EPRI Report TR-108026 Volumes 1,2 and 3 dated May 1997)

For additional information, please contact myself or our Consultant in the Washington, DC area, Dr. Rip G. Rice, President of RICE International Consulting Enterprises, 1331 Patuxent Drive, Ashton, MD 20861 (tel: 301-924-4224; fax: 301-774-4493; e-mail: RipRice_Ozone@compuserve.com). Dr. Rice was instrumental in the preparation of this petition and can respond quickly to requests, if necessary.

Yours very truly,

Dee M. Graham, Ph.D.

EPRI Food Technology Alliance

Energy Delivery & Utilization

Resolution Concerning the Use of Ozone in Food Processing

The Use of Ozone as an effective sanitizer and disinfectant worldwide beginning in France in 1902 and has been documented in an Expert Panel Report entitled "Evaluation of the History and Safety of Ozone in Processing Food for Human Consumption". This Declaration of GRAS Status for Use of Ozone in Food Processing was presented to FDA on April 10, 1997 and published thereafter in the scientific literature and the trade press.

Numerous ozone applications have been installed throughout the food industry in the United States during the past two years. The benefits to public food safety are major, especially related to the food hazards identified in the President's Food Safety Initiative. These include newer pathogens such as *E. coli* 0157:H7, *Listeri*a, and resistant cyst formers such as *Cryptosporidiu*m and *Giardia*, all of which are inactivated effectively by ozonation.

We urge the responsible Federal Agencies, particularly USDA-FSIS and FDA-CSAN to proactively support the adoption of ozonation for application broadly in food and agriculture as described in the Expert Panel Report Declaration of GRAS Status for ozone on April 10, 1997 and in this Petition.

Resolution Signers

Dennis Lavelle, President
Dell Industries
3428 Bullock Lane
San Luis Obispo, CA 93401

William P. Roenigk
Senior Vice President,
National Chicken Council
1015 Fifteen Street, NW – Ste. 930
Washington, DC 20005-2605

James T. C. Yuan, Ph.D.
Head, Food & Biochemical Research
Air Liquide
5230 South East Avenue
Countryside, Illinois 60525

Caleb L. Gilchrist, Ph.D.
Director, Scientific Affairs
American Meat Institute
1700 North Moore Street, Ste. 1600
Arlington, VA 22209

Charles D. Sopher, Ph.D.
Director, EPRI
Food & Agricultural Technology Alliances
2000 L Street, Suite 805
Washington, DC 20036

Nari Nayini, Ph.D.
Senior Development Associate
Food Applications R&D
Praxair, Inc.
7000 High Grove Boulevard
Burr Ridge, Illinois 60521-7595

Robert E. Smith, Ph. D., President
R. E. Smith Consulting, Inc.
222-B Eagle Point Road
Newport, Vermont 05855

Jurgen Strasser, Ph.D., President
Process & Equipment Technology
3312 Las Huertas Road
Lafayette, CA 94549-5109

Richard Forsythe, Ph.D.
Professor Emeritus – Poultry Science Dept.
University of Arkansas
Fayetteville, Arkansas 72701

Charles W. Pearsall
Vice President
RGF Environmental Group
3875 Fiscal Court, Suite 100
West Palm Beach Florida 33404

Barbara Blakistone
Senior Specialist
Food Chemistry & Packaging Dept.
National Food Processors Association.
1350 I Street, NW, Ste. 300
Washington, DC 20005

Stuart Proctor, Jr.
National Turkey Federation
1225 New York Ave NW-Ste 400
Washington, DC 20005

Michael W. Pariza, Ph. D.
University of Wisconsin – Madison
Food Research Institute
1925 Willow Drive
Madison, WI 53706

S. R. Tatini, Ph.D.
Food Sciences & Nutrition Dept.
University of Minnesota
1334 Eckles Avenue
St. Paul, MN 55108-6099

Lee C. Ditzler
President
Novazone
346 Earhart Way
Livermore, CA 94550

Dee M. Graham, Ph.D., President
R and D Enterprises
2747 Hutchinson Court
Walnut Creek, CA 94598

Frank Busta, Ph.D.
Professor and Emeritus Dept. Head
Food Sciences & Nutrition
University of Minnesota
1334 Eckles Avenue, Room 258
St. Paul, MN 55108-6099

Abit Massey
Georgia Poultry Federation
P. O. Box 763
Gainesville, Georgia 30501

Don Dalton
U.S. Poultry & Egg Association
1530 Cooledge Road
Tucker, GA 30084

Rip G. Rice, Ph. D.
President
Rice International Consulting Enterprises
1331 Patuxent Drive
Ashton, MD 20861

Sharon P. Shoemaker, Ph.D.
Executive Director
California Institute of Food &
Agricultural Research
250 Cruess Hall
Davis, CA 94516

Don Dalton
U. S. Poultry and Egg Associ
1530 Cooledge Road

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1.0 IDENTITY AND TECHNICAL PROPERTIES OF OZONE

1.1 IDENTITY AND COMPOSITION (WOJTOWICZ, 1996)

Ozone, O3, (Food Chemicals Codex FCC IV July 1996, p. 277; Chemical Abstracts Service No. [10028-15-6]) is an allotropic form of oxygen first recognized as an unique substance in 1840 (Schönbein, 1840). Its characteristic pungent odor which is present in areas close to operating electrical equipment (photocopying machines, movie projectors, UV bulbs, etc.) is readily detectable by human olfactory senses at low levels. It is thermally unstable, meaning that it autodecomposes rapidly to produce oxygen (from which it is produced). This means that the material cannot be produced centrally, bottled, stored, and shipped. Instead, ozone must be produced (generated) and applied at its point of use.

The ozone molecule is composed of three atoms of oxygen, each linked together so that neither oxygen atom is free of the other two. However, since ozone is produced by physically rupturing an oxygen molecule to split it into atoms, one of which combines with a stable oxygen molecule, it is known in the art that one of the oxygen atoms comprising the ozone molecule is more active chemically than the other two, when it is released from the two other oxygen atoms, which again revert to stable molecular oxygen.

Ozone is the strongest oxidant and disinfectant readily available for the treatment of air, water and wastewater. These attributes, plus the fact that ozone decomposes to form the innocuous byproduct oxygen, make ozone ideal for the treatment of air, water and wastewaters. Indeed, the most commercially significant application of ozone is in the treatment of drinking water, which began in Europe in 1906. By early 2000, there were well over 3,000 such water treatment installations all over the world, but primarily in Europe. However, the USA now boasts well over 300 potable water treatment plants in operation using ozone (Rice et al., 2000).

Other major commercial applications for ozone, which rely on its oxidation and disinfection capabilities, include treatment of bottled waters, swimming pool waters, cooling tower waters, municipal and industrial wastewaters, odor control, and pulp bleaching. In Japan, ozone is used extensively in the food industry for cleanliness and preservation in food processing plants, as well in household refrigerators to control odors.

1.2 PHYSICAL, CHEMICAL AND OTHER PROPERTIES

1.2.1 Physical Properties Including Stability

At ambient (room) conditions, ozone is an unstable, bluish-colored gas which has a gram-molecular weight of 48.0, a gas density of 2.144 grams/liter at 0EC and atmospheric pressure, and a boiling point of minus 112EC at atmospheric pressure (Pryor and Rice, 2000). It is only partially soluble in water but generally more so than oxygen -- see Table 1.01. It has a characteristic pungent odor detectable by the human olfactory senses at concentrations as low as 0.01-0.05 ppm in ambient air, and at even lower concentrations by use of appropriate analytical instrumentation (Pryor and Rice, 2000). Ozone is a powerful oxidizing agent, having an

oxidation potential of 2.07 volts in alkaline solutions. It is therefore capable of oxidizing many types of organic and inorganic materials.

Table 1.01. Solubility of Ozone And Oxygen in Water (Pryor and Rice, 2000)

Temperature, EC	O_3 Solubility, mg/L 3% O_3 in gas phase	O_2 (from air), Solubility mg/L
0	20	6.9
20	8.9	4.3

Ozone is relatively unstable in aqueous solutions. It has been reported repeatedly in early literature to have a half-life in distilled water of 20-30 minutes at 20EC before reverting back to simple diatomic oxygen (O_2), and that range remains a good rule-of-thumb for “clean” waters. However, in real-world waters, such as those encountered in food processing plants, potable water treatment plants and the like, readily oxidizable organic and inorganic contaminants normally are present, and most of these will react rapidly with dissolved ozone, thereby decreasing the half-life of ozone. Consequently, in real-world waters, the half-life of dissolved ozone can range from a few seconds (in "dirty" waters) to many minutes and even hours (in cleaner waters).

In addition, some water quality parameters, in particular, temperature and pH, affect the decomposition rate of ozone, and therefore its half-life. As these two parameters increase, so does the decomposition rate of ozone, decreasing its apparent half-life.

Ozone has been shown to be explosive if produced in concentrations at 48 wt. percent in oxygen (Thorp, 1955; Warakomski, 1994). This high concentration, however, generally is never produced by commercially available ozonation equipment used today in the food industry. Much lower concentrations of ozone gas are produced for industrial or municipal applications by corona discharge ozone generators, generally at levels of 1-3% (w/w) from air and 2-12% (w/w) from oxygen. Consequently, there should be no concern about explosivity of ozone in currently available ozone generation equipment.

1.2.2 Chemical Properties (Hoigné, 1988)

In pure water, ozone decomposes at temperature- and pH-dependent rates by a series of complex free radical chain reactions to produce, among other transient species, the hydroxyl free radical. At pH below 6.5 and at cold temperatures, the decomposition rate of ozone is very slow, and molecular ozone (O_3) is the dominant species in aqueous solution. However as pH rises above 8 or so and as water temperature increases, the rate of ozone decomposition also increases. Above pH 10 (at any temperature at which water is a liquid), for example, the rate of ozone decomposition is practically instantaneous. The decomposition rate also can be accelerated by the presence of hydrogen peroxide or ultraviolet radiation.

The hydroxyl free radical itself is a more powerful oxidizing agent than is molecular ozone, having an oxidation potential of 2.83 V (compare O_3 at 2.08 V). This makes possible the oxidation of organic materials that are only slowly oxidized by molecular ozone. On the other

hand, hydroxyl free radicals are transient in nature, having a half-life measurable only in microseconds. Because of its short half-life, concentrations of hydroxyl free radicals cannot rise above very low levels, on the order of 10^{-12} molar (Glaze and Kang, 1989a,b). Processes that involve the deliberate generation of hydroxyl free radicals (by coupling ozone with high pH, hydrogen peroxide, or UV radiation) to oxidize refractory organic materials have been termed "Ozone Advanced Oxidation Processes" (Ozone AOPs).

Once formed, the hydroxyl free radical is subject to a variety of rapid reactions. Not only are the reaction rates of hydroxyl free radicals with organic materials very fast (on the order of 10^{13} sec^{-1}), but the radicals are rapidly quenched (destroyed) by the presence of carbonate and bicarbonate anions. These latter two species are components of natural alkalinity in aqueous, real-world solutions.

Although reaction rates of molecular ozone are rapid, they can be much slower and more selective than reaction rates of the hydroxyl free radical. For example, the reaction rate of phenol with molecular ozone is very fast (on the order of 10^{6} sec^{-1} -- still 10 million times slower than reaction rates with hydroxyl free radicals). On the other hand, the reaction rate of acetate ion (or acetic acid) with molecular ozone is very slow (on the order of 10^{-6} sec^{-1}). Fortunately, advanced oxidation is available to increase the destruction rate of most ozone-refractory organics such as acetate ion.

In natural waters (real-world waters) the presence of naturally occurring organic materials as well as alkalinity anions promote the decomposition of ozone and scavenging of the produced hydroxyl free radicals, respectively. The presence of natural organics will increase the ozone demand of the water being treated. The presence of alkalinity anions will remove hydroxyl free radicals and decrease the advanced oxidation possibilities. Consequently, to derive the maximum disinfection and oxidation effects from ozone, the gas should be applied to as clean a water as is practicable under the specific circumstances.

1.2.3 Byproducts of Ozone

1.2.3.1 Organic Byproducts (Glaze, 1986 Rice and Gomez-Taylor, 1986)

Oxidation of organic materials by ozone, regardless of reaction rates involved and regardless of the simultaneous presence of hydroxyl free radicals, proceeds in stages, each stage producing one or more simpler (in some cases more complex) byproducts. If oxidation is provided in sufficient quantity and for sufficient time periods, the many organic materials can be totally oxidized to carbon dioxide and water. However, total oxidation is rare, particularly in most commercial applications, in which the treatment objective usually is to destroy some offensive parameter (odor, color, a specific microorganism or some particular contaminant). As a result, many new oxidation byproducts can be formed during ozonation or ozone advanced oxidation processes.

The toxicology of ozone was reviewed by Newell (1997), concluding that the oxidation products formed when foodstuffs are treated with ozone are similar to those formed when water is treated with ozone. A National Toxicology Program report (NTP, 1994) addressed the toxicology and carcinogenicity of ozone, reporting 4-week studies with mice, and 2-year and lifetime studies

with rats. The report concluded that under conditions of these 2-year and lifetime inhalation studies, there was no evidence of carcinogenic activity of ozone in male or female F344/N rats exposed to 0.12, 0.5, or 1.0 ppm of ozone.

For the most part, the organic byproducts of ozone oxidation can be lumped together as aldehydes, ketones, acids, alcohols, and mixed functionality derivatives, e.g., keto-acids, aldehyde-acids, keto-aldehydes, aldehyde-alcohols, and the like. In turn, these types of compounds are much more readily biodegradable than are the starting compounds prior to ozone oxidation. In the water treatment industry it is well-known that if ozonation is the terminal step in the water treatment plant and treated water is sent to distribution systems without a residual of chlorine, considerable regrowth of microorganisms (feeding on ozone-oxidized organic byproducts) can occur. To prevent this regrowth, most water treatment plants employing ozone provide a biological filtration step after ozonation in the water treatment process, prior to adding a small residual of chlorine or chloramine. With granular activated carbon as the filter medium, and after development of the appropriate biomass on the GAC surface, the ozone-produced organic byproducts can be mineralized (converted biochemically to carbon dioxide and water) in a short time (less than 5 minutes empty bed contact time).

The biofiltration step produces biologically stable water, and the concomitant low amount of residual chlorine compound required to produce a residual stable throughout the distribution system provides a finished water with much lower tastes. Consequently, to avoid the presence of organic ozone-produced byproducts in food processing applications, this same technique can be followed.

1.2.3.2 Inorganic Byproducts

Any inorganic material capable of being oxidized can be oxidized by ozone to its highest valence state. Ferrous iron is converted rapidly to ferric ion, which in water, quickly hydrolyzes to produce ferric hydroxide. Manganous ion quickly oxidizes to form the insoluble manganese dioxide – however continued ozonation can convert this into the soluble permanganate anion. Nitrite anion rapidly produces nitrate anion upon ozonation. Sulfide ion first forms elemental sulfur, but continued ozonation produces sulfite, sometimes sulfur trioxide, and ultimately sulfate anion. Cyanide anion is oxidized rapidly by ozone to cyanate anion, which slowly hydrolyzes to produce nitrogen gas and carbon oxides.

Ozone oxidation of bromide ion in water is chemically complex and can lead to the formation of brominated organics as well as bromate anion. The first step in the oxidation of bromide ion (by ozone or by chlorine) is the formation of hypobromous acid/hypobromite ion (so-called “free bromine”). Hypobromous acid, in turn, is an excellent brominating agent, and is the source of mixed bromo-chloro-organics when chlorine is added to waters containing natural organics.

Ozonation of hypobromite anion can produce bromate anion, a material shown by Japanese and U.S. EPA researchers to cause cancers in laboratory test animals when fed in sufficient quantities. Consequently, the U.S. EPA has promulgated a Maximum Contaminant Level for bromate ion of 10 Φg/L (U.S. EPA, 1998).

Chlorine does not oxidize hypobromite ion to produce bromate anion. Neither does ozone oxidize hypobromous acid to produce bromate ion. On the other hand, bromate ion has been found in concentrated solutions of sodium hypochlorite used for treated drinking water supplies.

There are three techniques currently available to minimize the production of bromate ion during ozone treatment of drinking waters, and these are being practiced today:

1. **Ozonate at pH 6.5 or below.** At pH 6.5, all of the oxidized bromine species is present as hypobromous acid. Since there is no hypobromite ion present, bromate ion cannot be formed.

2. **Minimize the concentration of residual ozone during ozonation.** The lower the level of residual ozone, the more competitive are the non-hypobromite water components for the ozone, and the less bromate ion can be formed.

3. **Add traces of ammonia to water prior to ozonation.** Ammonia will react with hypobromous acid, effectively removing it from the system being ozonated. Hypobromite will equilibrate with hypobromous acid, which will be removed quickly by reacting with more ammonia to produce monobromamine. The process continues until the concentration of hypobromous acid has been minimized as well as the level of hypobromite anion. On the other hand, this technique is problematic, in that after a finite period of time (dependent on at least pH and temperature), the rate of ozone oxidation of monobromamine becomes significant, reforming bromide ion to reenter the oxidation cycle. Consequently, this last technique requires a maximum ozone contact time of about 10 minutes to be effective.

1.2.4 Ozone Manufacturing Processes

Ozone can be generated on-site as required by several techniques, three of which are available commercially at the present time – corona discharge, UV radiation and electrolysis.

1.2.4.1 Corona Discharge

The most commercially significant technique is by corona discharge (the so-called "silent electrical discharge" procedure). This is tantamount to producing synthetic lightning. In a corona discharge ozone generator, the feed gas (dried air, oxygen, or mixtures thereof), passes between two closely spaced electrodes (one of which is coated with a dielectric material) under a nominal applied potential of ~10 kV. A silent or barrier discharge occurs when the gas becomes partially ionized, resulting in a characteristic violet glow when air is the feed gas (with high purity oxygen the violet coloration is seldom observed).

Efficiency of ozone production by corona discharge depends, for the most part, on the strength of microdischarges (which is influenced by a number of factors such as the gap width, gas pressure, properties of the dielectric and metal electrodes, power supply, and the presence of moisture). In weak discharges, a significant fraction of the energy is consumed by ions, whereas in stronger discharges, almost all of the discharge energy is transferred to electrons responsible for the formation of ozone. The optimum is a compromise that avoids energy losses to ions but at the same time obtains a reasonable conversion efficiency of oxygen atoms to ozone.

If air is used as the feed gas, it must be scrupulously dried and be free of traces of oils and greases (oxidized by ozone). Moist air gives rise to nitrogen oxides in the ozone generator which will form nitric acid which will corrode the generator, requiring frequent maintenance and down time. If liquid oxygen provides gaseous high purity oxygen, the moisture problem (and necessity to dry air) is avoided.

The process of electrically rupturing oxygen atoms to produce oxygen ions or atoms that combine with more oxygen to produce ozone also liberates considerable heat. This heat generated during the process must be removed from the generator to avoid the reverse reaction (ozone reverting to oxygen) from taking over and decreasing the efficiency of ozone generation by this technique. Consequently cooling of the corona discharge ozone generator becomes a critical component in generator design. Normally cooling is provided by water, but there are air-cooled CD-ozone generators commercially available, especially on smaller scale, appropriate for many food applications.

Properly designed and operated CD-ozone generators commercially available today are capable of producing kg/h quantities of ozone in gas phase concentrations of 1-5% by weight in air and up to 14% by weight in high purity oxygen.

1.2.4.2 Ultraviolet Radiation

The mechanism of photochemical production of ozone is similar to that which occurs in the stratosphere, that is, oxygen atoms formed by the photo-dissociation of oxygen by short wavelength UV radiation (# 240 nm) react with oxygen molecules to form ozone. Although the theoretical quantum yield of ozone by this technique is ~2, in practice the actual yield is more on the order of ~ 0.5, because the low pressure mercury lamps produce not only the 185 nm radiation responsible for the production of ozone, but also the 254 nm radiation that destroys ozone. Medium pressure UV that produces higher levels of 185 nm radiation produces more ozone.

An advantage of generating ozone by UV radiation is that ambient air can be used efficiently as the feed gas. On the other side, quantities of ozone generated per 40-W UV bulb are low (ca 0.5 g/h) at maximum concentrations of 0.25 wt %. However, these maximum ozone yields and concentrations cannot be attained simultaneously by the UV method. The low concentrations of ozone available from UV generators limit their applicability for water treatment to special applications. However, their use to generate ozone for air treatment can be effective.

1.2.4.3 Electrolysis

High current density electrolysis of aqueous phosphate solutions at room temperature produces ozone and oxygen in the anodic gas. Electrolysis of 68 wt-% sulfuric acid can produce 18-25 wt-% ozone in oxygen when a well-cooled cell is used. Although electrolysis of water can produce high concentrations of ozone, the output is low, and the cost is several times more than that of the corona discharge process. However, small electrolytic units are being used commercially for treatment of ultra-high purity waters in pharmaceutical and electronic industries.

1.2.5 Application of Ozone

1.2.5.1 In the Aqueous Phase

Because ozone is only partially soluble in water, efficient transfer of ozone into solution requires the dispersion of gaseous ozone into small bubbles. This is accomplished in various types of positive pressure ozone contractors such as bubble diffuser/bubble columns, mechanically agitated vessels, turbine mixers, tubular reactors, in-line static mixers as well as negative pressure reactors (venturi) and injectors. In positive pressure devices, ozone gas under pressure exiting the ozone generator is forced through small apertures supported under the water. With negative pressure devices, ozone gas is drawn into the flowing water stream to be treated. Under these mixing conditions, bubbles are sheared and mixed thoroughly with the aqueous fluid, decreasing the liquid film thickness but increasing both interfacial area and contact time.

Plate and packed columns also can be employed to increase the gas-liquid contact.

Single or multiple contact chambers can be used, each with ozone contact times ranging from two up to 20 minutes, depending on the application. Faster ozone mass transfer rates result in faster disinfection and (usually) oxidation rates. However, slow-to-oxidize organics are unaffected by increased ozone mass transfer rates, and advanced oxidation techniques should be considered for these types of materials.

1.2.5.1.1 Off-Gas Treatment

Ozone transfer efficiencies vary with the number of contacting stages and typically are above 90%. However, since even a 95% ozone absorption efficiency can result in a contactor off-gas containing as much as 740 ppm (by wt) of ozone (based on 1.5 wt-% ozone in air feed gas), treatment is required to reduce the ozone concentration to an acceptable maximum level for discharge to the local environment. This can be accomplished thermally (300-350EC for $\exists$ 5 sec) and/or by catalytic means, and sometimes (for low concentrations and/or small volumes) by passage through wet granular activated carbon beds. This latter method (wet GAC) is ***NOT*** recommended for large-scale ozone destruction, due to the large amount of heat that is liberated upon ozone destruction, which can build up sufficiently to cause the GAC medium to deflagrate.

1.2.5.2 Application in the Gas Phase

Reaction rates of contaminants in air are much lower (slower) in the gas phase than in aqueous phase. Additionally, the option of adjusting pH is not open in air spaces, although increasing the relative humidity is an effective option.

Pumping ozone gas from the generator into an air space to be treated is the simplest approach, and is most effective when the air contaminants to be treated are rapidly reactive with ozone – e.g., many odoriferous compounds such as hydrogen sulfide, molds, spores, and some airborne microorganisms. For those air contaminants that are only slowly affected by ozone, the accepted procedure is to draw contaminated air into an enclosed structure in which ozone is mixed with the contaminated air for such period of time as is necessary to destroy (or inactivate) the contaminant(s). At the outlet of the air/ozone contactor, excess ozone is destroyed, and the

cleaned and decontaminated air is recirculated to its intended enclosure or discharged to the ambient atmosphere.

1.2.6 Application Of Adjuncts With Ozone

With the advent of ozone advanced oxidation, there are recent instances reported of coupling ozone with either hydrogen peroxide or with ultraviolet radiation, techniques that are designed to promote the formation of hydroxyl free radicals with the stated objective of increasing the amount of microbiocidal activity above that of ozone itself. However, all information developed to date indicates that the half-life of hydroxyl free radicals in water is only microseconds in length, and that the maximum concentration of hydroxyl free radicals that has been measured in aqueous solution is very low, on the order of 10^{-12} M. These facts tend to indicate that there can be no microbiocidal benefit of hydroxyl free radical over that provided by molecular ozone. Nevertheless some recent reports indicate what appears to be a synergy – or an increased amount of microbial inactivation by applying these combinations to certain foodstuffs over what is obtained by applying ozone or UV radiation alone. For example, Naitoh (1992) investigated synergistic sporicidal activities of gaseous ozone and UV irradiation. The author reported that combined treatment reduced the contact time required for the inactivation.

For completeness, these reports will be summarized in this petition.

1.2.6.1 Ozone + Hydrogen Peroxide

With this combination of oxidants in aqueous solution, both agents destroy each other, and both agents give rise, eventually, to hydroxyl free radicals. It is customary procedure to add the requisite amount of hydrogen peroxide to solution and then pass that solution through an ozone contacting apparatus. Ozone reacts immediately with hydrogen peroxide in solution, and if the amount of ozone dosed in the contactor is always greater than the amount of peroxide initially added to solution, at the outlet of the contact chamber it will not be possible to measure a level of residual ozone in solution.

In advanced oxidation practice, it has been learned that for optimum oxidative performance, each pollutant that needs to be destroyed requires a specific weight ratio of peroxide to ozone. For example, in potable waters, the taste- and odor-causing compounds geosmin and 2-methylisoborneol are destroyed rapidly with peroxide to ozone weight ratios of about 0.3 to 1. On the other hand, refractory pesticides sometimes require weight ratios as high as 0.8 to 1 (peroxide to ozone). Consequently, it is advisable when evaluating the use of this advanced oxidation process to first determine which polluting constituents of the water or wastewater requiring treatment are present, and then to determine experimentally the optimum range of peroxide to ozone weight ratio required for their destruction.

Glaze et al. (1987) have shown that if the weight ratio of peroxide to ozone rises above 1:1, the rates of oxidation of organics in water actually slow down. This means that if excess hydrogen peroxide is present over the amount of ozone added, at least some of the advantages of advanced oxidation are lessened. It also means that there will be no molecular ozone present at any time during ozone contacting for microbial disinfection.

1.2.6.2 Ozone + UV Radiation

With this combination of agents, it is customary to place a UV bulb (or multiple bulbs) in the ozone contacting chamber. As water flows through the chamber, first the UV bulb(s) is(are) turned on and ozone is added. As long as the amount of UV radiation dosed is in excess of the amount of ozone present, all ozone will be converted instantaneously to decomposition products, ending rapidly as hydroxyl free radicals. With this setup, a measurable residual ozone level is impossible to find. However, if the UV bulb(s) is(are) turned off during the process but the ozone addition is continued, then a dissolved ozone level is built up rapidly. Turning the UV bulb(s) on again instantly destroys any residual ozone.

With this type of advanced oxidation process, there is always UV radiation present over the amount of ozone being dosed, but no molecular ozone will be present. According to present theories, any antimicrobial activity observed then must be due to the presence of UV radiation.

1.2.6.3 UV Radiation + Hydrogen Peroxide

Although this procedure does not involve ozone, the two agents also produce hydroxyl free radicals in solution, and rapidly. As with the ozone/hydrogen peroxide system, the peroxide is added to solution and the so-treated water or wastewater then is passed through a reactor chamber containing the appropriate number of UV bulbs. Since the amount of UV radiation is designed to be in excess of the amount of peroxide added initially, all of the peroxide will be converted to hydroxyl free radicals -- by the same token there will always be excess UV radiation applied to the solution. According to present theories, any antimicrobial activity observed would be a result of the UV radiation, and not the presence of the hydroxyl free radicals generated.

In fact, Kruithof and Kamp (1999) have conducted advanced oxidation experiments on drinking waters spiked with certain pesticides and with *Clostridia* spores. Solutions treated by ozone alone showed little oxidative benefit for destroying the pesticides. However, ozone/peroxide (excess peroxide/ozone, therefore, no residual ozone could be formed) rapidly destroyed the pesticides tested. The same pesticides also were destroyed rapidly when treated by the UV/peroxide approach.

When solutions were spiked with *Clostridia* spores (22,000 CFU/mL) and treated under optimum ozone/peroxide and ozone/UV conditions for pesticide destruction, it was found that the ozone/peroxide lowered the microorganism levels to 21,000 CFU/mL – however the UV/peroxide system lowered the microorganism levels to essentially zero/mL. This is confirmation that:

1. There was very little antimicrobial effect provided by hydroxyl free radicals (in the ozone/peroxide system)

2. There was very little molecular ozone present in the ozone/peroxide system (excess peroxide over ozone so as to optimize pesticide oxidation), consequently there was little beneficial effect of the system on lowering microorganism levels

3. Because excess UV radiation was present in the UV/peroxide system, microorganism levels were lowered rapidly – by the UV radiation present, and not by the hydroxyl free radicals.

Despite the theories and the work of Kruithof and Kamp (1999), several reports have been made of an apparent increase in antimicrobial activity in some food applications when ozone is combined in water with either peroxide or UV radiation. These will be discussed in the next section.

1.2.7 Antimicrobial Properties of Ozone

1.2.7.1 General Comments (Wojtowicz, 1996, p. 980)

Ozone is a more effective broad-spectrum disinfectant than chlorine-based compounds (105). Ozone is very effective against bacteria because even concentrations as low as 0.01 ppm are toxic to bacteria. Whereas disinfection of bacteria by chlorine involves the diffusion of HOCl through the cell membrane, disinfection by ozone occurs with lysing (i.e., oxidative rupture) of the cell wall. Disinfection rates depend on the type of organism and are affected by ozone concentration, temperature (106), pH, turbidity, the presence of ozone-oxidizable materials, the tendency (or not) for the microorganisms to form clumps, and the type of ozone contactor employed (Zhu et al., 1989). The presence of ozone-oxidizable substances in water exerts an ozone demand, and this can retard disinfection until the initial ozone demand has been satisfied, at which point rapid disinfection is observed.

The antimicrobial activity of ozone is based essentially on its powerful oxidizing effect, which causes irreversible damage to the fatty acids in the cell membrane and to cellular macromolecules, such as proteins, and DNA (Fettner and Ingols, 1959; Hoffman, 1971; Naitoh, 1994). This action is particularly effective in air at high relative humidity, the bacteria being killed by ozone more readily in the swollen state than when dry.

Gram-positive bacteria are more sensitive to ozone than are gram-negative bacteria.

1.2.7.2 In Water

Kim et al. (1999)

Inactivation mechanisms. Reactions of ozone with various chemical compounds in aqueous systems occur in two different and coexisting modes, one involving direct reactions of molecular ozone and the other being a free radical-mediated destruction mode (Staehelin and Hoigné, 1985). Singlet oxygen is a likely intermediate reactive species in the biochemical damage caused by ozone (Kanofrsky and Sima, 1991). These multiple mechanisms also may apply to the destructive effect of ozone on bacteria. However, Hunt and Marinas (1997) recently found that *E. coli* was inactivated primarily by molecular ozone.

Giese and Christenser (1954) suggested that the bacterial cell surface is the primary target of ozone activity. Scott and Lesher (1963) detected the leakage of cell contents with ozone treatment. They proposed the double bonds of unsaturated lipids in the cell envelope as the primary site of attack. Murray et al.. (1965) assumed that lipoprotein and lipopolysaccharide

layers of gram-negative bacteria would be subjected first to attack by ozone that results in a change in cell permeability, eventually leading to lysis.

According to Komanapalli and Lau (1996), viability of *E. coli* K-12 was unaffected by short-term exposure (1-5 min) to 600 ppm ozone gas but membrane permeability was compromised. With longer exposures, up to 30 min, cell viability decreased, with a progressive degradation of intracellular proteins. According to Bancroft and Richter (1931), ozone causes cellular proteins to flocculate. Bringman (1955) suggested that chlorine selectively destroyed certain enzymes, whereas ozone acted as a general protoplasmic oxidant. Sykes (1965) concurred with Bringman (1955) about the cause of cell destruction by ozone. Ingram and Haines (1949) found a general destruction of the dehydrogenating enzyme systems in *E. coli* after treatment with ozone and proposed that death of the cell may result from interference with the respiratory system.. Barron (1954) suggested that the oxidation of sulfhydryl groups (SH- to S-S) in the enzyme is the principal cause of death. Ozone caused a more rapid decrease in P-galactosidase activity in the cytoplasm than alkaline phosphatase activity in the periplasm of *E.. coli* (Takamoto et al., 1992).

Ozone may inactivate microorganisms by causing damage to their genetic material. In studies by Prat et al. (1968) and Scott (1975) on DNA of *E. coli*, the pyrimidine bases were modified by ozonation, with thymine being more sensitive to ozone than cytosine and uracil. Different mechanisms were proposed to explain the inactivation of viruses by ozone. Kim et al. (1980) examined tritiated f2 bacteriophage and its RNA after exposure to ozone. RNA was released from the phage particles during ozonation, and the treated phage had reduced infectivity for spheroplasts. Electron microscopic examination showed that the phage coat was broken by ozonation into many protein subunits and that the specific adsorption of the phage to host pili was inversely related to the extent of phage coat breakage. Roy et al. (1981), however, observed that the damage to the viral nucleic acid is the major cause of the inactivation of poliovirus 1 (Mahoney). Ozone not only damaged the viral RNA but also altered polypeptide chains of the viral protein coat.

Inhibitory spectrum: bacteria. Ozone inactivates numerous bacteria that include gram-negative and gram-positive and both vegetative cells and spore forms (Table 1.02). It is not feasible to compare the sensitivity of bacteria to ozone using results from different sources; effectiveness of ozone varies appreciably with minor changes in experimental variables. Selected studies, however, are presented to illustrate the effectiveness of ozone against various bacterial species.

Table 1.02. Inactivation of Bacteria by Ozone (Kim et al., 1999)

Bacterium	Inactivation, (log_{10})	Treatment time (min)	Concentration (mg/L)	pH	Temp. (EC)	Medium	Reactor type	Reference
Bacillus cereus	*>2.0*	*5*	0.12		28	O_3 demand-free water		Broadwater et al., 1973
B. cereus (spores)	*>2.0*	*5*	*2.29*		28	O_3 demand-free water		ditto
Escherichia coli	*4.0*	1.67	0.23-0.26	7	24	O_3 demand-free water	Continuous flow	Farooq & Akhlaque, 1983
E. coli	*3.0*	19	Initial *2.2* residual *0.06*	*7.5*	*16*	Raw wastewater	Continuous flow	Joret et al., 1982
E. coli	2.0	0.1	0.53	6.8	1	Phosphate buffer	Batch	*Fetner & Ingols, 1956*
Legionella pneumophila	>4.5	20	0.32	7	24	Distilled water	Batch	*Edelstein et al., 1982*
Mycobacterium fortuitum	1.0	1.67	0.23-0.26	7	24	O_3 demand-free water	Continuous flow	Farooq & Akhlaque, 1983
Pseudomonas fluorescens	*>2.0*	*0.25*						*Burleson et al., 1975*
Salmonella enteritidis	1.0	*0.25*	*8%* (wt/wt)		*25*	Broiler carcass	Ozone gas	*Ramirez et al.,* 1994
Salmonella typhimurium	4.3	1.67	0.23-0.26	7	24	O_3 demand-free water	Continuous flow	Farooq & Akhlaque, 1983
Staphylococcus aureus	*>2.0*	0.25		7	*25*	Phosphate buffer	Batch (bubbling)	*Burleson et al., 1975*

Finch et al. (1988) determined the extent of inactivation of *E. coli* using ozone doses of 4.4 to 800 Φg/liter at contact times of 30 to 120 sec. They reported 0.5- to 6.5-log decreases in counts of *E. coli*, depending on the ozone dose and contact time. *Pseudomonas putrefaciens* was added to a pilot-scale water recycling system where ozone was maintained at 1.5 ppm (1995). The population of *P. putrefaciens* decreased 3-logs after 5 min and 6-logs after 20 min of exposure. Bactericidal action of ozone depends on the medium in which bacteria are present. Dave et al. (1998) showed that a *Salmonella enteritidis* population, in distilled water, decreased 6-logs at a low concentration of ozone (1.5 ppm). However, when broiler skin was inoculated with *Salmonella enteritidis* and exposed to an ozone-air mixture (8%, wt/ wt) for 15 sec, approximately 1-log reduction in population of the pathogen was observed (Ramirez et al., 1994). Antimicrobial effects of ozonated water in a recirculating concurrent reactor, against different bacterial species, were evaluated (Restaino et al., 1995). Death rates among the gram-negative bacteria (*Salmonella typhimurium*, *E. coli*, *P. aeruginosa*, and *Yersinia enterocolitica*) were not significantly different, whereas among gram-positive bacteria, *L. monocytogenes* was significantly more sensitive than either *Staphylococcus aureus* or *Enterococcus faecalis*. Kim (1998) determined the effectiveness of ozone against foodborne microorganisms such as *P. fluorescens*, *Leuconostoc mesenteroides*, *L. monocytogenes*, and *E. coli* O157:H7 in a batch-type reaction system. He found that all tested microorganisms were inactivated by 1.5- to 5-logs at 1

to 1.5 ppm of ozone within 15 sec. Among these microorganisms, *L. monocytogenes* was the least resistant and *L. mesenteroides* was the most resistant to ozone (see Figure 1.01 (Kim et al., 1999).

When compared to vegetative cells, bacterial spores have greater resistance to ozone. Broadwater et al. (1973) reported that the lethal threshold concentration for *Bacillus cereus* was 0. 12 mg/liter, while that for *E. coli* and *B. megaterium* was 0. 19 mg/liter. The threshold concentration for the spores of *B. cereus* and *B. megaterium* was 2.3 mg/liter.

When ozone treatment was combined with other deleterious factors, greater inactivation rates of bacterial spores were observed. Foegeding (1985) found that acidic pH enhanced the lethality of ozone against the spores of *Bacillus* and *Clostridium*. The author also suggested that the spore coat is a primary protective barrier against ozone. Naitoh (1992) found that the addition of metallozeolites, ascorbic acid, and isoascorbic acid improved the inactivation of *B. subtilis* spores by ozone treatment at 5 to 50 ppm for 1 to 6 h. Naitoh (1992) also investigated synergistic sporicidal activities of gaseous ozone and UV irradiation. The author reported that combined treatment reduced the contact time required for the inactivation.

Inhibitory spectrum: fungi. Ozone is an effective fungicidal agent (see Table 1.03; Kim et al., 1999). Ewell (1938) stated that depending on cleanliness, minimum continuous concentrations of 0.6 to 1.5 ppm ozone (in air) were necessary to prevent mold growth on eggs kept at 0.6EC and 90% relative humidity (RH), whereas 2.5 to 3.0 ppm ozone were required to control molds on beef stored under similar conditions. According to Farooq and Akhlaque (1983), ozone also inactivated yeast. The population of *Candida parapsilosis* decreased by 2-logs in 1.67 min when the yeast was exposed to 0.23 to 0.26 mg/liter ozone. Counts of *C. tropicalis* decreased by 2-logs when the yeast cells were exposed to ozone at 0.02 mg/liter for 20 sec or at 1 mg/liter for 5 sec (Kawamura et al., 1986).

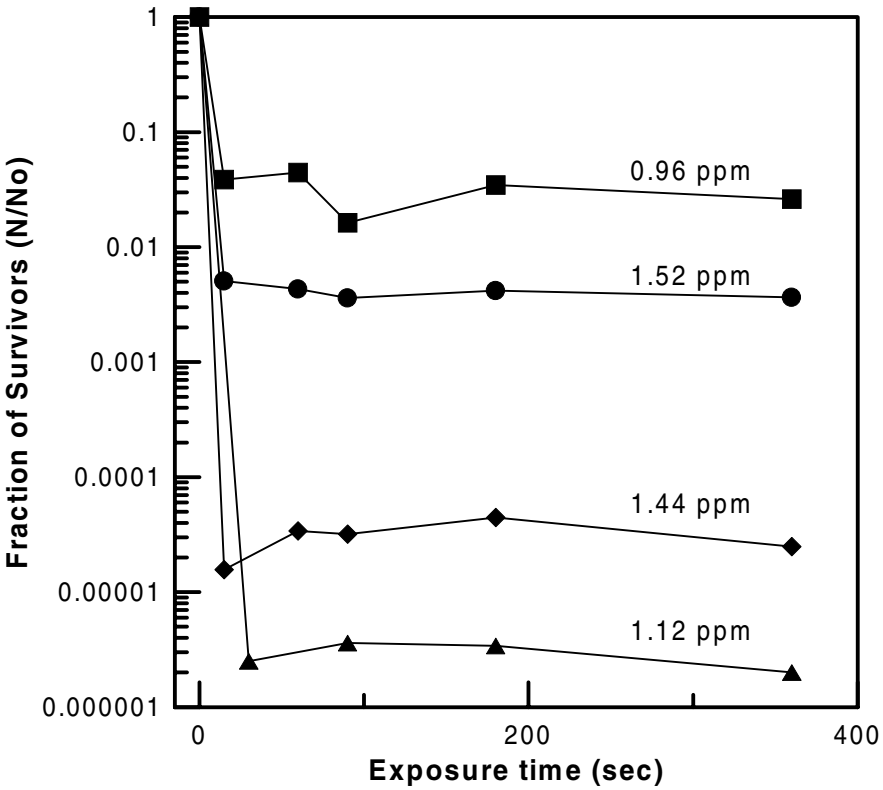


Figure 1.01. Inactivation of foodborne microorganisms by 0.96 to 1.5 ppm ozone at pH 6.0 and 25EC. *E. coli* O157:H7: *P. fluorescens*; *L. mesenteroides*; , *L. monocytogenes* (Kim et al., 1999).

Table 1.03. Inactivation of Yeasts by Ozone (Kim et al., 1999)

Yeast	Inacti-vation, (log10)	Treat-ment time (min)	Concen-tration (mg/L)	pH	Temp. (EC)	Medium	Reactor type	Refer-ence
Candida parapsilosis	*2.7*	*1.67*	0.23-0.26	7	24	O_3 demand-free water	Continuous flow	Farooq & Akhla-que, 1983
C. Tropicalis	*2.0*	*0.30-0.08*	0.02-1.0	7.2	20	O_3 demand-free water	Continuous flow	Kawa-mura et al., 1986

Yeasts appear more sensitive than molds to ozone treatments. More than 4.5-logs of *C albicans* and *Zygosaccharomyces bailii* populations were killed instantaneously in ozonated water in a recirculating concurrent reactor, whereas less than 1-log of *Aspergillus niger* spores were killed after a 5-min exposure (Restaino et al., 1995). The average ozone output level in the deionized water was 0.188 mg/liter. Naitoh and Shiga (1982) found that the threshold of microbiocidal activity of aqueous ozone (0.3-0.5 mg/liter) against spores of *Aspergillus*, *Penicillium*, and *C. paracreus* was 90 to 180, 45 to 60, and 5 to 10 min of exposure, respectively.

Inhibitory spectrum: viruses. Ozone is potentially an effective virucidal agent (Table 1.04, Kim et al., 1999). Relatively low concentration of ozone and short contact time are sufficient to inactivate viruses. However, inactivation of viruses in wastewater requires longer contact time and larger ozone concentrations than inactivation in ozone demand-free systems because of oxidizable materials present in the medium. Majumdar et al. (1973) reported a rapid decrease in virus survival at ca. 1 mg/liter initial ozone concentration after a 2-min contact period. Katzenelson et al.. (1974) demonstrated the potent virucidal effect of ozone and suggested that ozone alone or in combination with chlorine be used in treating water and wastewater.

Herbold et al. (1989) tested the resistance of viruses and bacteria to ozone in steadily flowing water at 20EC and pH 7. The order of resistance was poliovirus 1 < *E. coli* < hepatitis A virus < *Legionella pneumophila* serogroup 6 < *B. subtilis* spores. For the complete inactivation of poliovirus 1 and hepatitis A virus (ca. 10^4 $TCID_{50}$/mL), 0.13 and 0.25 to 0.38 mg/liter ozone was needed, respectively. Emerson et al.. (1982) found that viruses associated with cells or cell fragments are protected from inactivation by ozone at concentrations that readily inactivate purified virus. The authors tested ozone to disinfect human epithelial cells infected with poliovirus (Sabin type) or coxsackievirus A9. In a continuous-flow ozonation system, the cell-associated poliovirus and coxsackievirus samples demonstrated survival at applied ozone dosages of 4.06 and 4.68 mg/liter, respectively for 30 sec. Unassociated viruses in the control treatment were inactivated by 0.081 mg/liter for 10 sec. Ultrasonic treatment did not increase inactivation of the cell-associated enteric viruses. In a batch reactor, inactivation of cell-associated viruses required 2 min contact with 6.82 mg/liter and ozone residual of 4.7 mg/liter, whereas unassociated viruses were completely inactivated after 5 min with 4.82 mg/liter and ozone residual of 2.18 mg/liter.

Inhibitory spectrum: protozoa. Table 1.05 (Kim et al., 1999) lists results of studies on inactivation of some protozoa by ozone. Wickramanayake et al. (1984) reported the effect of aqueous ozone on the inactivation of cysts of *Naegleria gruberi* and *Giardia muris*. The *N. gruberi* cysts were more resistant to ozone than *G. muris*. A 2-log decrease of population was

observed with 0.2 mg/liter ozone at 25EC and pH 7 in 7.5 min for *N. gruberi* compared to 1.05 min for *G. muris*. The intestinal parasite, *Cryptosporidium parvum*, that can cause gastroenteric disease was exposed to ozone that inactivated >90% of the parasite population within 1 min at 1 mg/liter ozone in ozone demand-free water (Korich et al., 1990).

Environmental factors. Although microorganisms inherently vary in sensitivity to ozone, the physiological state (e.g., the stage of growth) and environmental factors affect greatly the degree of inactivation of these microorganisms by ozone. Susceptibility of microorganisms to ozone varies according to the pH of the medium, temperature, humidity, additives (e.g., acids, surfactants, and sugars), and the amount of organic matter surrounding the cells.

Table 1.04. Inactivation of Viruses by Ozone (Kim et al., 1999)

Virus	Inacti-Vation, (log10)	Treat-ment time (min)	Concen-Tration (mg/L)	pH	Temp. (EC)	Medium	Refer-ence
Bacteriophage f2	0.7	10	0.1	7.2	20	Activated sludge effluent	Harakeh & Butler, 1985
Bacteriophage f2	>4.3	0.16	0.41	7	20	Water	Boyce et al., 1981
Coxsackie virus B5	4.0	2.5	0.4	7.2	20	Sludge effluent	Harakeh & Butler, 1985
Coxsackie virus A9	1.7	0.16	0.035	7	29	Water	Boyce et al., 1981
Enteric virus	> 1.7	29	Initial 4.1, residual 0.02	7.8	18	Raw wastewater	Joret et al., 1982
Hepatitis A virus	2.7	0.02	0.25	7.2	20	Phosphate buffer	Herbold et al., 1989
Human rotavitus	0.7	10	0.31	7.2	20	Sludge effluent	Harakeh & Butler, 1985
Poliovirus type 1 (Mahoney)	2.5	1.67	0.23-0.26	7	24	O_3 demand-free water	Farooq & Akhlaque, 1983
Poliovirus type 1 (Mahoney)	1.0	0.53	0.51	7.2	20	Water	Roy et al., 1981
Poliovirus type 1	2.0	10	0.2	7.2	20	Activated sludge effluent	Harakeh & Butler, 19854
Vesicular stomatitis	>2.0	0.25		7	25	Phosphate buffer	Burleson et al., 1975

Table 1.05. Inactivation of Protozoans by Ozone (Kim et al., 1999)

Protozoan	Inactivation, (log_{10})	Treatment time (min)	Concentration (mg/L)	pH	Temp. (EC)	Medium	Reactor type	Reference
Cryptosporidium parvum	> 1.0	5	1	7	25	O_3 demand-free water	Batch	*Korich et al., 1990*
Giardia lamblia	2.0	1.1	0.7	7	5	water	Batch	Wickramanayake et al., 1984
G. muris	2.0	2.8	0.5	7	5	water	Batch	ditto
Naegleria gruberi	2.0	2.1	2.0	7	5	water	Batch	ditto

Temperature. A decrease in the temperature of an aqueous medium results in increased solubility of ozone. On the other hand ozone decomposition is accelerated with increasing temperature. Herbold et al. (1989) reported that ozone effectiveness on hepatitis A virus and *E. coli* diminished when the temperature increased from 10E to 20EC. However, Katzenelson et al. (1974) indicated that lowering the temperature from 5 to 1EC had a minor effect on the inactivation kinetics of microorganisms.

pH. The stability of aqueous ozone increases by decreasing the pH. Researchers attributed the rapid decomposition of ozone in aqueous solutions with high pH to the catalytic activity of the hydroxyl ion (Adler & Hill, 1950; Hewes and Davison, 1973). Leiguarda et al. (1949) reported that bactericidal efficiency of ozone on *E. coli* and *C. perfringens* was slightly greater at pH 6.0 than at pH 8.0. Farooq et al. (1977) noted higher a survival rate of *Mycobacterium fortuitum* during ozone treatment when pH was increased. The authors attributed this increased survival to a smaller ozone residual as the pH of water increased. Foegeding (1985) studied ozone inactivation of *Bacillus* and *Clostridium* spores at different pH values and found that acidic pH values enhanced the lethality of ozone.

Humidity. Elford and Ende (1942) used low ozone concentrations and long exposures at variable relative humidity to disinfect airborne microorganisms. At RH <45%, the germicidal power of ozone was negligible. Inactivation was substantial even at concentrations far below 0. 1 mg/liter when high humidity was used. Ewell (1946) demonstrated that microorganisms were killed more readily by ozone in an atmosphere having a high rather than low RH. The need for moisture in a cell for it to be inactivated by ozone was elucidated by Guerin (1963). The author indicated that not only were desiccated microorganisms more resistant than hydrated cells to sterilization by ozone, but once desiccated, some cells were difficult to rehydrate sufficiently to be susceptible to ozone sterilization. Guerin (1963) concluded that ozone was an effective inhibitor only for nondehydrated microorganisms. Kim and Yousef (1999) found a similar reaction of ozone in food ingredients containing natural contaminants. They treated a powdered food ingredient, having variable water activities (a_w), with gaseous ozone. When the a_w value of the ingredient was ca. 0.95, 10^2 to 10^5 CFU/g were inactivated with 200 ppm ozone in an ozone-oxygen mixture. However, similar ozone concentration had no effect on the microbial load of products with a_w less than 0.85. In order to counteract this microbial resistance to ozone, water was added to the powder and the mixture was shaken by an orbital shaker at 25EC overnight. This treatment increased a_w from 0.85 to 0.95 and the total count by 1-log. When the rehydrated product (ca. 8 x 10^3 CFU/g) was treated with ozone, more than 2-logs were inactivated by 200

ppm and the total count was less than 10^1 CFU/g (the detection limit) when 300 ppm ozone was used.

Ozone demand of the medium. Having a high oxidation potential, ozone reacts with microorganisms fast, resulting in high lethality. Kim (1998) observed a 2- to 3-log reduction of *P. fluorescens*, *E. coli* O157:H7, *L. mesenteroides*, and *L. monocytogenes* in <10 sec of exposure to <1 ppm ozone in a pure cell suspension system. However, ozone also reacts with other particles and compounds if placed in an environment such as food systems that are rich in organic matter. The effectiveness of ozone depends on the amount applied, but more so on residual ozone in the medium after demands have been met. Venosa (1972) pointed out that one of the most serious failures by various investigators has been their inability to distinguish between the concentration of applied ozone and residual ozone necessary for effective disinfection. Therefore, the ozone availability and the decay of ozone during the course of the experiments should be reported, otherwise underestimation of the actual ozone dose used in the experiments to affect the inactivation may follow. Yang and Chen (1979) reported that the bactericidal effects of ozone were lower in Ringer solution, 5% NaCl solution, and in the presence of egg albumin than in distilled water. Restaino et al. (1995) reported that in the presence of organic material, death rates of some gram-positive microorganisms (e.g., *S. aureus* and *L. monocytogenes*), and gram-negatives, *E. coli* and *Salmonella typhimurium*, in ozonated water were not significantly affected by 20 ppm of soluble starch but were significantly reduced by addition of 20 ppm of bovine serum albumin. Residual ozone in water containing bovine serum albumin was significantly lower than in deionized water and water with soluble starch.

When microorganisms are suspended in an ozone demand-free medium, the only source of ozone demand is the seeded organisms. In water, ozone may react directly with dissolved substances, or it may decompose to form secondary oxidants that react immediately with solutes. These different pathways of reactions lead to different oxidation products, and they are controlled by different types of kinetics. The solutes present in water influence appreciably the rate of the radical-type chain reactions leading to the decomposition of ozone. This reaction is promoted by solutes, such as formic acid and methanol, that convert the nonselective hydroxyl (EOH) into a superoxide (EO_2^-) radical that is a more efficient chain carrier. Such promoters counteract the inhibiting effects of EOH radical scavengers that generally terminate the chain reaction. Acetic acid and acetate are known to terminate the reaction by scavenging EOH, thus stabilizing ozone in aqueous solutions (Forni et al., 1982; Hoigné and Bader, 1976; Sehested et al., 1987). Schuchmann and Sonntag (1989) explained ozone effectiveness in reducing the load of organic matter (added D-glucose) in raw water purification. They found that the direct mode of reaction by ozone predominated at high glucose concentration, however, the EOH pathway predominated at low glucose concentration, especially at higher pH (e.g., 9.0).

Ozone accessibility to targeted microorganisms. Most microorganisms may not be found in free suspension as discrete particles, especially when they are present in food systems. The association of microorganisms or subcellular components with suspended matter may hamper the accessibihty of ozone to microorganisms. Longley et al. (1978) pointed out that such criteria as degree of mixing and mass transfer must be considered to establish the efficacy of ozone for a particular disinfection application.

Berg et al. (1964) used the ultrasonic treatment to breakdown clumps of microorganisms and thus increased the antimicrobial effect of ozone dramatically. Burleson et al. (1975) reported that ozone and sonication resulted in a synergistic effect on the inactivation of viruses and bacteria in secondary effluent. They reasoned that sonication may enhance interphase transport, break up particulate organic material and clusters of bacteria, and produce cavitation to reduce the high surface tension caused by organic matter. However, Kim and Yousef (1998) could not confirm the effectiveness of sonication during treatment of fresh lettuce with ozone. Sonication may enhance the decomposition of ozone or increase ozone demand by detaching organic materials from the cut surfaces of the shredded lettuce.

Kim and Yousef, 2000

The objectives of this investigation were to (a) measure the ability of ozone to inactivate selected food-borne microorganisms, (b) develop a method to study kinetics of microbial inactivation by ozone, and (c) use the kinetics data to predict the inactivation of microorganisms over a broad range of ozone concentrations.

Abstract: Ozone was tested against *Pseudomonas fluoroscens, Escherichia coli* O157:H7, *Leuconostoc mesenteroides,* and *Listeria monocytogenes*. When kinetic data from a batch reactor were fitted to a dose-response model, a 2-phased linear relationship was observed. A continuous ozone reactor was developed to ensure a uniform exposure of bacterial cells to ozone and a constant concentration of ozone during the treatment. Survivors plots in the continuous system were linear initially, followed by a concave downward pattern. Exposure of bacteria to ozone at 2.5 ppm for 40 seconds caused a 5 to 6 log decrease in count. Resistance of tested bacteria to ozone followed this descending order: *E. coli* O157:H7, *P. fluorescens, L. mesenteroides,* and *L. monocytogenes*.

Materials and Methods

Preparation of inoculum: *P. fluorescens* ATCC 17386, *E. coli* O157:H7 ATCC 35150, *L. mesenteroides* subsp. *mesenteroides* ATCC 14935, *L. monocytogenes* Scott A were used in this study. Stock cultures of these bacteria were stored at -20EC in suitable broth media supplemented with 10% (v/v) glycerol. Inoculum of *L. monocytogenes* Scott A was prepared as described by Lou and Yousef (1996). The stock culture of *L. monocytogenes* was inoculated into trypticase soy broth supplemented with 0.6% Bacto yeast extract and the mixture was incubated at 35EC for 24 h; this was followed by two additional successive transfers under similar conditions. Bacterial cells were harvested by centrifugation at 3,000 x g in a refrigerated (4EC) centrifuge and washed twice in 0.1M phosphate buffer solution (pH 7) to a final concentration of 1-3 x 10^9 CFU/mL. Similar protocols were used to prepare inocula of the other bacteria, but growth media and incubation conditions were different. *E. coli* O157:H7 was sub cultured twice in trypticase soy broth (TSB) and incubated at 35EC for 24 h. *P. fluorescens* was grown in nutrient broth (Difco) and incubated at 26EC for 24 h. *L. mesenteroides* was cultured in Lactobacilli MRS broth (Difco) and incubated at 26EC for 24 h. Inoculum. sizes of different bacteria were estimated by measuring absorbance at 600 nm (A_{600}) and calculating approximate counts from standard curves for absorbance in contrast to bacterial count.

Preparation of Aqueous Ozone: Ozonated water was obtained by bubbling ozone about 2.5% (v/v, in oxygen carrier gas) into a round-bottom flask containing about 1000 mL, sterile deionized water at 25EC. The flow rate of ozone into the flask was controlled by a peristaltic pump. A stainless-steel sparger with 10 Φm pore size was used for bubbling ozone into the water. The ozonated water was circulated by a peristaltic pump through Norprene tubing to the spectrophotometer's flow cell (0.6 mL capacity, with a light path of 1-cm. The spectrophotometer was used to continuously measure ozone absorbance at 258 nm. The rate of ozone flow was predetermined to achieve and maintain the desired equilibrium ozone concentration. Equilibrium was attained when absorbance at 258 nm remained relatively constant.

Inactivation Studies: A batch and two continuous reaction systems were setup to study the inactivation of selected microorganisms by ozone. Because of the reactivity of ozone, only glass containers and tubes and Norprene tubing were used. The pH and temperature were kept constant during the treatment, but ozone concentration (or dosage) and contact time were varied.

Batch Reaction System: Batch reaction simply involves mixing a predetermined volume of ozonated water and cell suspension in a container and neutralizing the mixture after it is held for a given time. Ozonated water (95 mL) was transferred to a reaction vessel, and ozone concentration was determined by absorbance measurement. The vessel was inoculated with 5 mL cell suspension (in 0.05 M phosphate buffer) to attain a count of about 10^8 CFU/mL. Vessel contents were stirred using a Teflon-coated magnetic stirrer bar at 100 rpm. Samples (5 mL each) of the reaction mixture were taken at intervals, and each was mixed immediately with 0.5 mL neutralizing solution (0.005M sodium thiosulfate) to halt the reaction. Cells were counted for all samples by the standard plate count method. In some experiments, ozonated water (9.5 mL) was mixed with 0.5 mL cell suspension, and the mixture was held for 30 s before the ozone neutralizer (0.5 mL) was added to stop the reaction. In this case, samples of ozonated water were taken immediately before adding cell suspensions to determine ozone concentration by the indigo method.

Continuous Reaction System: Two continuous reactors (designated I and II) were setup as follows:

Reactor I. The reactor was designed to mix continuously the ozonated water and the cell suspension in a y-shaped type tube. When ozone concentration reached a steady state, the ozonated water and the cell suspension, in separate reservoirs, were pumped out at similar flow rates (17.4 mL/min) by a peristaltic pump having two identical pump heads. These two streams were mixed in a y-shaped Norprene tubing (internal diameter = 1.6 mm), and the mixture was carried through the tubing, which has seven sampling ports (3-way valves) at different lengths. Samples of equal size were collected from ports and mixed immediately with thiosulfate solution (1.24 g/L added at 10% of the sample volume) to neutralize residual ozone. Contact time(s) was calculated as follows: [resident volume between the mixing point and a sampling port / flow rate (mL/min)j x 60. The calculated contact time ranged from 0.4 to 37.8 s. Survivors were counted in collected samples.

Reactor II: A membrane filter (pore size, 0.45 Φm; diameter = 25 min; composition, mixed cellulose acetate and nitrate was mounted on the fritted glass base of a glass filtration unit, and

the unit was assembled. The funnel functions as a reservoir for the ozonated water. Cell suspension (1 to 2 mL, about 10^8 CFU/ mL) and subsequently ozonated water (1 to 10 mL) were drawn through the filter at a constant rate (22 mL/min) using a peristaltic pump. Contact time(s) was calculated as follows: {Volume of applied ozonated water / flow rate (mL/min)} x 60. Dosage was varied by using variable volume (1 to 10 mL) of ozonated water or by applying equal volumes of water that contained different concentrations of ozone. The filter with the treated cells was transferred into peptone water for ozone neutralization and cell detachment by vigorous agitation. Dilutions of samples and plate counting were performed.

Neutralization: Sodium thiosulfate (0.005M) neutralizer stock solution was prepared by dissolving 1.24 g of $Na_2S_2O_3.5H_2O$ in 1-L of freshly autoclaved distilled water. The amount of sodium thiosulfate solution was var ied depending on the estimated ozone concentration in the solution being neutralized. This neutralizer does not have an interfering absorbance at 258 nm, nor an adverse effect on treated microorganisms.

Microbiological Tests: For the enumeration of *P fluorescens, E. coli* O157:H7, and *L. mesenteroides*, nutrient agar (NA), tryptic case soy agar (TSA), and *Lactobacilli* MRS (MRS) were used, respectively. *L. monocytogenes* was enumerated on trypticase soy agar supplemented with 0.6% yeast extract.

Results

Inactivation Studies: Batch Reaction: The range of ozone concentrations varied, for practical reasons, when different microorganisms were tested. Sensitivities of treated bacteria were compared at similar ozone concentrations or by using dose-response plots. Sensitivities of *E. coli* O157:H7 and *Pseudomonas fluorescens* to ozone were slightly different. Ozone at 0.2 ppm inactivated 0.9 log of *P. fluorescens* in 30 s, whereas 1.2 ppm decreased the population by 5 log in a similar treatment time (Figure 1.02a). When *E. coli* O157:H7 was treated with 0.3 and 1.0 ppm ozone, the count decreased 1.3 and 3.8 log, respectively, in 30 s (Figure 1.02b).

Counts of *Leuconostoc mesenteroides* decreased by 1.3 and 3.3 logs when initial ozone concentrations were 0.3 and 1.5 ppm, respectively (Figure 1.02c). Ozone at about 4 ppm killed about 7 logs of *L. mesenteroides* CFU/mL. Ozone at 0.4 and 0.8 ppm, initially, inactivated 4.6 and 5.7 logs of *L. monocytogenes* CFU/mL (Figure 1.02d). Therefore, microorganisms tested in this study showed similar inactivation kinetics. Most inactivation occurred during the first 15 or 30 s of the treatment, and counts remained unchanged when the mixture was held for up to 6 min (results for the first 90 s only are shown in Figure 1.02).

Correlation between amounts of ozone remaining in the reaction mixture (residual ozone) and degree of inactivation was studied. A cell suspension of *L. mesenteroides* (about 10^7 CFU/mL was mixed with ozonated water to contain 0.3 to 2.1 pprn ozone, initially. The mixture was sampled to determine counts of survivors and the residual ozone (Figure 1.03a). When the initial ozone concentrations were 0.3, 1.1, and 2.1 ppm, residual ozone concentrations after 30 s, were 0.0, 0.5, and 0.9 ppm, respectively. Bacterial counts after 30 s of exposure were 2.2, <1 and <1 log CFU/mL, respectively (data not shown). Therefore, inactivation of bacteria by ozone is a rapid process; and this process continues until either survivors or residual ozone become

undetectable. Estimated ozone demand of 10^7 L. mesenteroides CFU/mL is 0.83 ppm. Therefore, about 10^9 molecule of ozone were used to inactivate each cell.

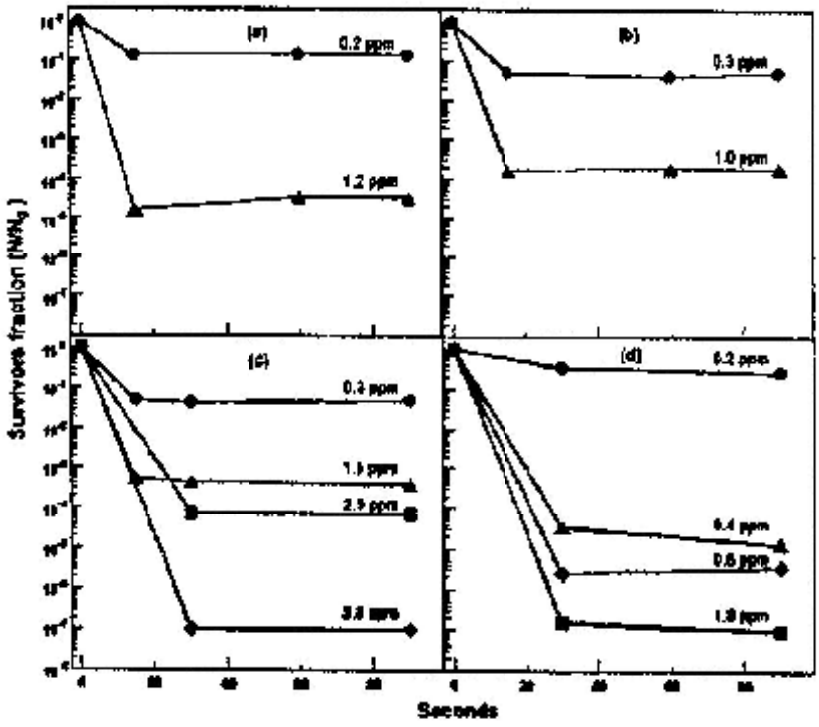


Figure 1.02. Survivors plots for the inactivation of (a) *P. fluorescens*, (b) *E. coli* O157:H7, (c) *Leu. mesenteroides*, (d) *L. mycocytogenes* (1.3×10^8 - 2.5×10^8 cfu/mL, initially) by ozone in the batch reactor at pH 5.9 and 25EC. N_0 = count of untreated sample; N = count of treated sample (Kim and Yousef, 2000).

Inactivation of *L. mesenteroides* by 1.3 ppm ozone was investigated when the initial count varied (Figure 1.03b). When initial inoculum sizes were 6.8, 7.1, 7.4, and 7.7 log CFU/mL, ozone concentration decreased by 62%, 58%, 83%, and 100%, respectively, during 15 s of treatment. A large decrease in residual ozone was observed initially, and the rate of decrease diminished when the mixture was held for up to 3 min (Figure 1.03b). A similar trend of inactivation kinetics was observed, that is, rapid decrease in count initially and minimal changes later (data not shown). Therefore, effectiveness of ozone varied considerably with inoculum sizes. Inactivation by 1.3 ppm ozone was less than 1-log for the largest inoculum (7.7 logs CFU/mL), but it was > 6-logs when an inoculum half the size of the former (7.4 logs CFU/mL) was used. Therefore, ratio between amounts of treated cells and added ozone should be considered carefully for maximum effectiveness of ozone.

In the batch reaction system, microorganisms are inactivated rapidly (< 30 s), and thus determination of inactivation kinetics technically is difficult (Figure 1.02). When the cell-ozone mixture was held for several minutes, no further change in count was observed. Thus, data relating this ultimate decrease in count or population inactivated (PI) in response to varying initial concentration of ozone were used to construct dose-response plots. *P fluorescens* PI was

plotted against initial ozone concentration (Figure 1.04a). Data were linear at two ranges of ozone concentrations. *P. fluorescens* PI values changed considerably with ozone concentration up to about 1 ppm. However, these values increased only moderately at concentrations > 1 ppm.. Similar inactivation kinetics were observed when *E. coli* O157:H7, *L. mesenteroides*, and *L. monocytogenes* were tested (Figures 1.04b to 1.04d).

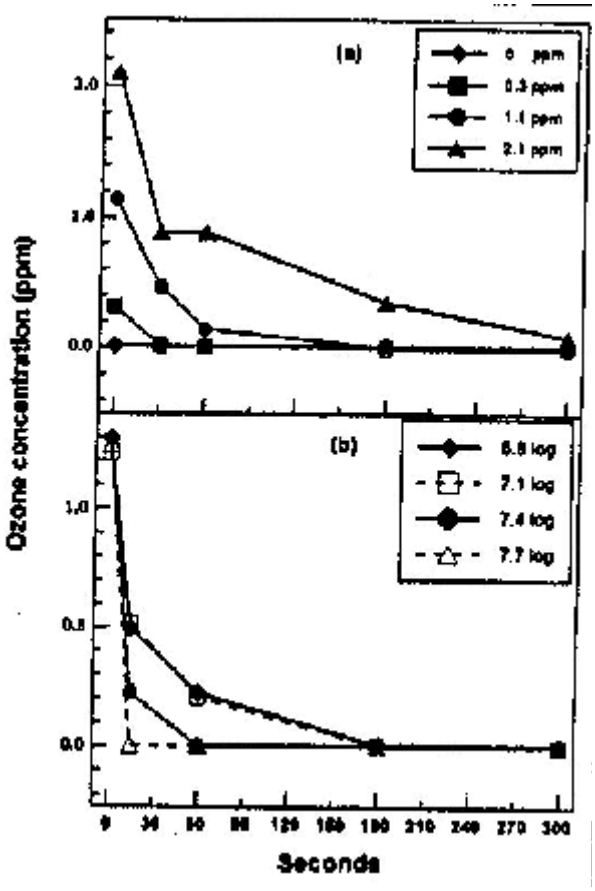


Figure 1.03. Changes of residual ozone concentration when variable initial ozone and *Leu. mesenteroides* cell concentrations (a) 10^7 cfu/mL and variable initial ozone; (b) variable inoculum size and initial ozone concentration of 1.3 ppm (Kim and Yousef, 2000).

PI-value at 1 ppm ozone was determined from equations describing the dose-response plots; these values are 4.6, 2.7, 3.5, and 7.5 logs for *P. fluorescens, E. coli* O157:H7, *L. mesenteroides*, and *L. monocytogenes*, respectively. *E. coli* O 157:H7 was the most resistant, while *L. monocytogenes* was the least resistant against ozone inactivation. Increase in PI values with increase in initial ozone concentration (that is, slope of the first segment of the dose-response plots) was greatest for *L. monocytogenes*. In conclusion, ozone concentration and cell inactivation are linearly- related over two ranges of ozone concentrations.

Continuous Reactions:

Reactor I: Unlike the batch system, this reactor permitted measuring bacterial inactivation after short periods (0 to 20 s) of exposure to ozone. Exposure to 0.1-0.7 pprn ozone caused 0.7 to 7.0 logs decrease in count, depending on the microorganisms. Most of the decrease in count occurred during the first 5 s of the treatment (Figure 1.05). Counts of *L. monocytogenes* decreased gradually as contact time increased (Figure 1.05d). This kinetic pattern is more distinctive than that seen in other bacteria. Resistance of *Listeria* against ozone was the least among the tested microorganisms; about 0.4 pprn ozone inactivated about 7 logs.

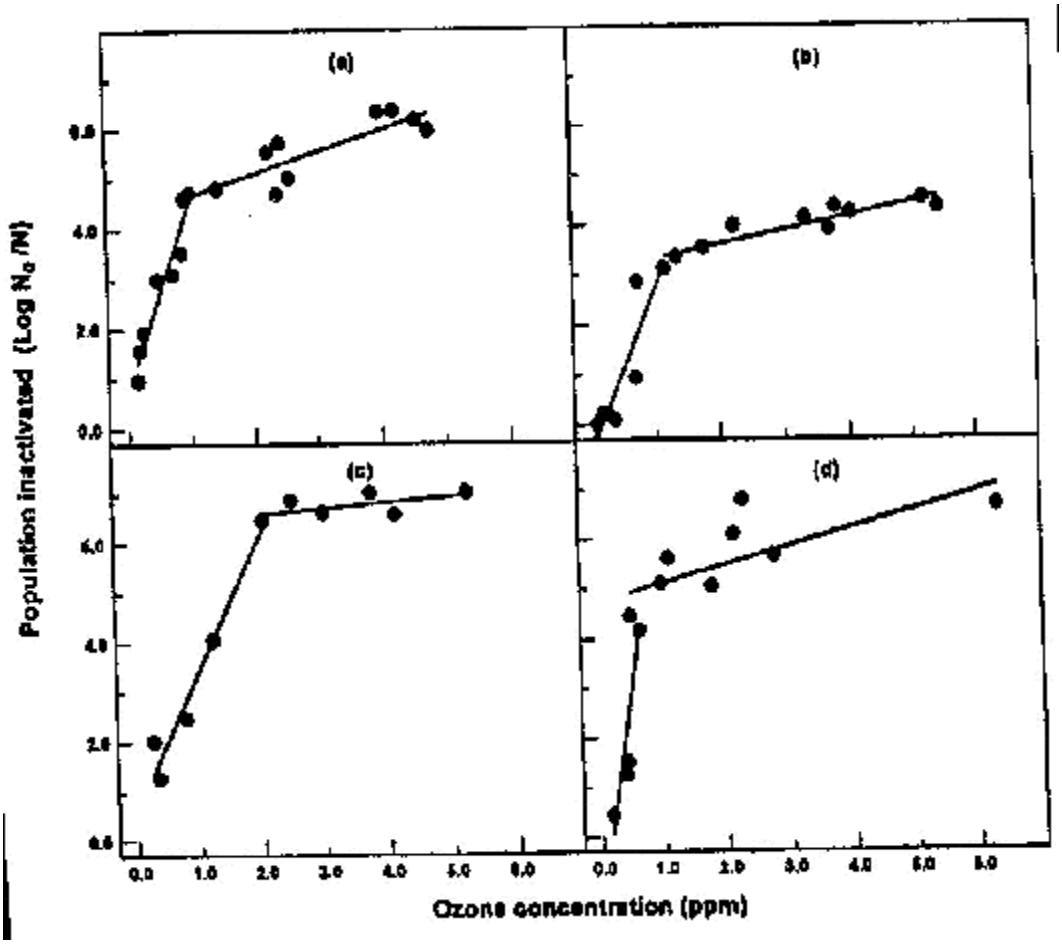


Figure 1.04. Dose-response plots for the inactivation of (a) *P. fluorescens*, (b) *E. coli* O157:H7; (c) *Leu. mesenteroides*; (d) *L. monocytogenes* (1.3×10^8 - 2.5×10^8 CFU/mL initially) by ozone in the reactor at pH 5.9 and 25EC. N_0 = count of untreated sample; N = count of treated sample (Kim and Yousef, 2000).

In spite of its advantages, this reaction system does not permit maintaining a constant ozone concentration during the treatment. After ozone and cell suspension meet in the y-tube, bacterial cells may consume ozone, and its concentration changes during the holding period. The reactor's design permits continuous mixing of fresh cells and ozonated water, but the reaction does not continue during the contact time. This may explain the similarity in the inactivation pattern in data obtained from this and the batch reaction system.

Reactor II: Inactivation kinetics were different with this reactor than with the previously tested systems. Survivors and dose-response plots were linear over a broader range of contact times and ozone concentrations, respectively. Inactivation of microorganisms by ozone also followed a concave downward curve at all ozone concentrations tested (Figure 1.06).

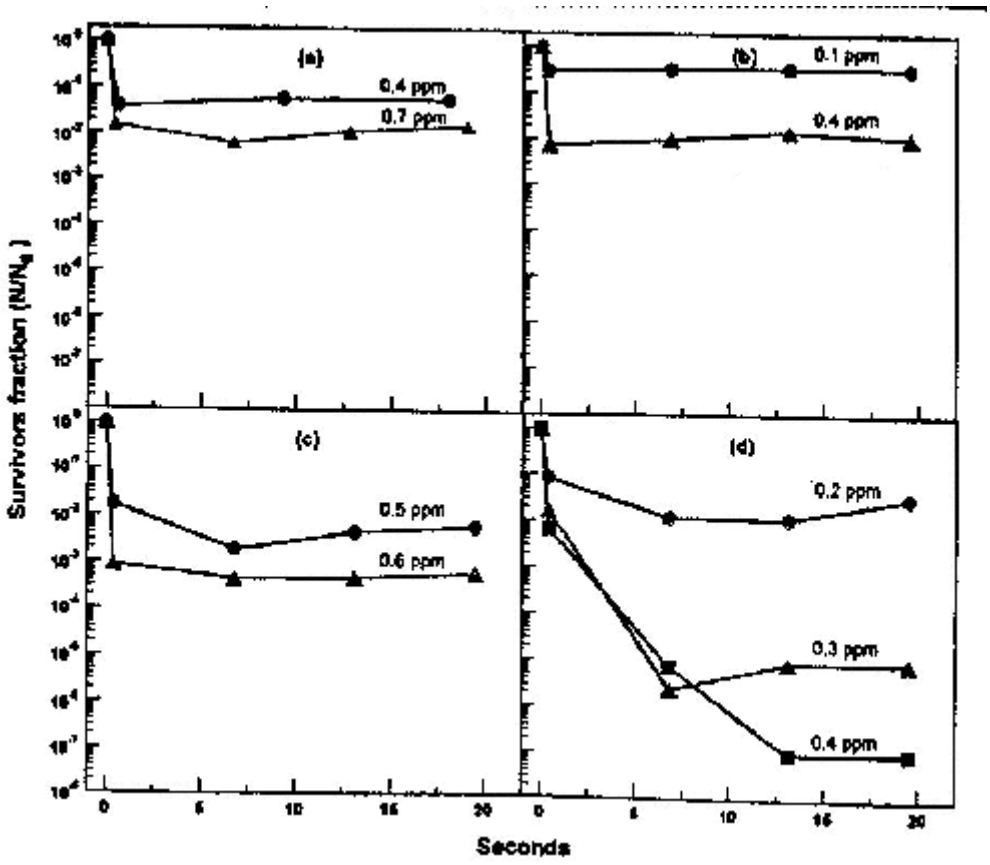


Figure 1.05. Survivors plots for the inactivation of (a) *P. fluorescens* (b) *E. coli* O157:H7; (c) *Leu. mesenteroides* (d) *L. monocytogenes* (9.6×10^7 - 4.3×10^8 CFU/mL, initially) by ozone in the continuous reactor I at pH 5.9 and 25EC. N_0 = count of untreated sample; N = count of treated sample (Kim and Yousef, 2000).

D-values were calculated from initial slopes of survivors plots, that is, during the first 5.4 to 8.5 s of exposure to ozone. D-values at different ozone concentrations were plotted against ozone dosage to construct log-log dose-response plots (Figure 1.07). Inactivation of some bacteria (for example, *P. fluorescens*) by ozone gave a scattered dose-response plot, but the relationship between D-value and ozone dosage was clearly linear. For the comparison of ozone resistance, D-values at 1 ppm ozone treatment for the tested microorganisms were calculated. They are 4.6 s for *P. fluorescens*, 6.2 s for *E. coli* O157:H7, 4.4 s for *L. mesenteroides*, and 3.3 s for *L. monocytogenes*. *E. coli* O157:H7 was the most resistant against ozone, while *L. monocytogenes* was the least resistant, which agrees with the results obtained from the batch and the continuous reactor I.

It should be cautioned, however, that low ozone concentrations were not tested in the continuous reactor II. Therefore, a different kinetic model may exist depending on the ozone concentration and the ozone-demanding substances present in the reaction.

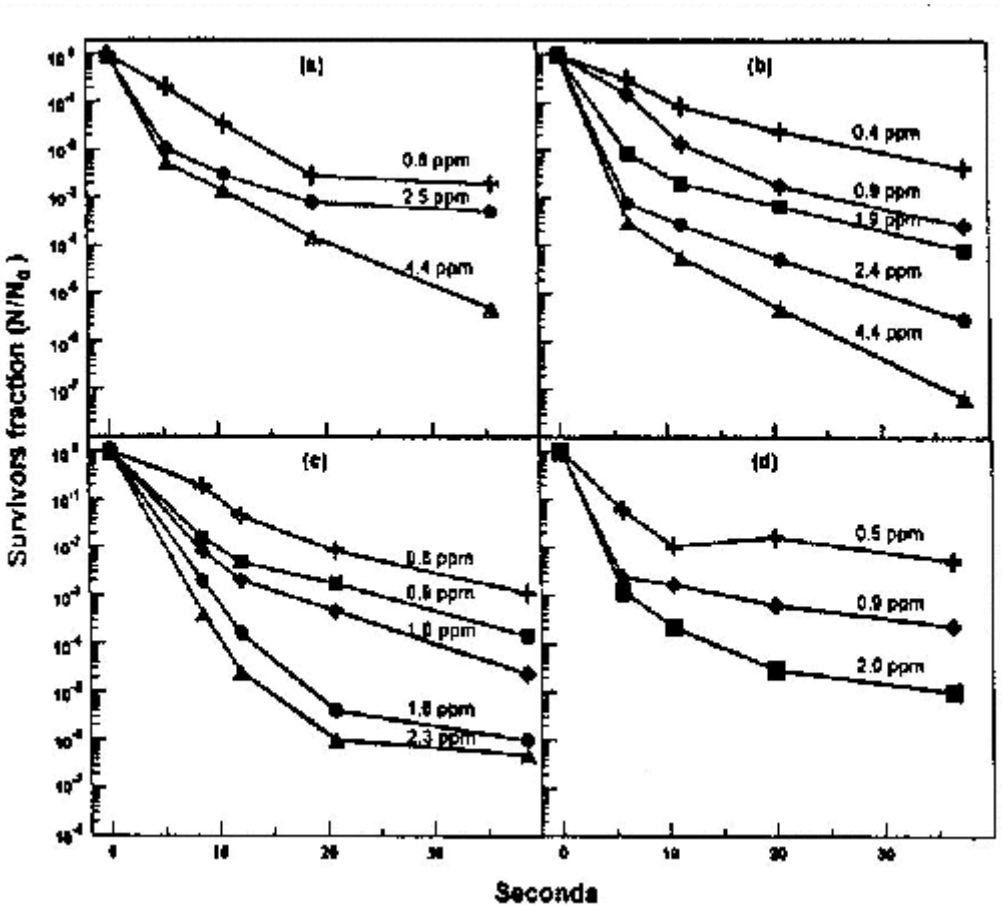


Figure 1.06. Survivors plots for the inactivation of (a) *P. fluorescens*; (b) *E. coli* O157:H7; (c) *Leu. mesenteroides* (d) *L. monocytogenes* (6.6×10^7 - 2.2×10^8 cfu/mL, initially) by ozone in the continuous reactor II at pH 5.9 and 25EC. N_0 = count of untreated sample; N = count of treated sample (Kim and Yousef, 2000).

Discussion

Data on inactivation kinetics by ozone vary appreciably among different research groups (Kim et al., 1999), however, our results are consistent with those reported by Finch et al. (1988). These authors used ozone (0.0044 and 0.81 mg/L) in 0.05 M phosphate buffer (pH 6.9) containing *E. coli* cells (about 10^7 CFU/mL). Bacterial count decreased by 3 to 6 logs in 60 s. The disinfection rate was fast initially. They concluded that the disinfection kinetics did not follow the pseudo first-order model that is normally assumed to approximate chemical disinfection of bacteria. In a batch-type reaction system, 0.065 mg ozone/L inactivated 3.5 log *E. coli* in 30 s (Katzenelson et al., 1974). A 2-stage action of ozone in the inactivation of *E. coli* was observed. The investigators concluded that ozone acts on microorganisms so quickly that it is practically impossible to measure the time required for 99% kill.

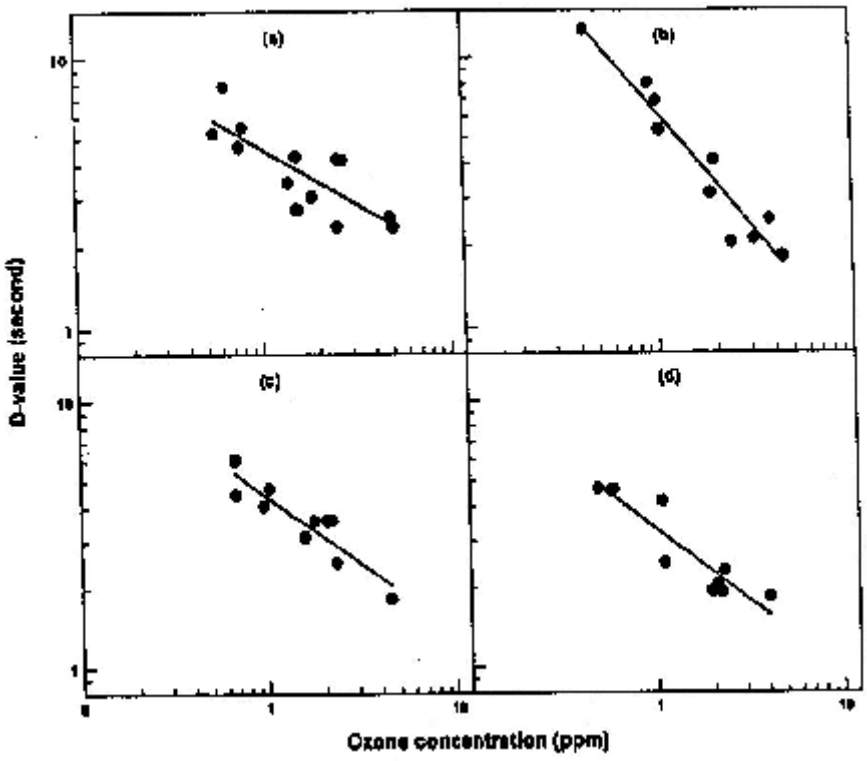


Figure 1.07. Log-log dose-response plots for the inactivation of (a) *P. fluorescens*; (b) *E. coli* O157:H7; (c) *Leu. mesenteroides* (d) *L. monocytogenes* (1.3×10^8 - 2.5×10^8 cfu/mL, initially) by ozone in the continuous reactor II at pH 5.9 and 25EC. N_0 = count of untreated sample; N = count of treated sample (Kim and Yousef, 2000).

Residual ozone was determined in order to reveal the mechanism involved in the kinetics in the batch reaction. Ozone decomposed very quickly while it reacted with microorganisms. When the microbial load was large relative to the amount of added ozone, residual ozone was not measurable, but bacterial survivors were detected. When the ratio of ozone to microbial load was large, residual ozone was detected, but the bacterial population was eliminated. Therefore, the extent of inactivation depends on the ozone demand of the added microbial load. Gomella (1972) stated that evidence of a trace ozone residual is an acceptable sign of complete disinfection in water treatment. However, Sommerville and Rempel (1972) reported the presence of coliforms in water containing 0.1 mg/L ozone residual. Farooq and others (1977) observed the degree of inactivation was profoundly affected by the initial organism population. These authors detected 4-logs reduction when the initial density of *Candida parapsilosis* was 1.4×10^5 CFU/mL, but observed no inactivation when the initial density was 1.6×10^7 CFU/mL.

The inactivation process is an interaction between ozone and the microorganism, analogous to a chemical reaction that follows the course of a first-order reaction (Shechter 1973). In an ozone demand-free reactor system, the only source of ozone demand is the seeded microorganisms. Ozone reacts with cells in the treated water, liberating new molecules capable of reacting with ozone. Therefore, ozone decreases chemical oxygen demand (COD), whereas it increases biochemical oxygen demand (BOD). Scott and Lesher (1963) reported that ozone caused

leakage of cell content into the medium and lysis of some cells. Therefore, ozone demanding substances are generated during the ozone inactivation process. Finch et al. (1988) found that 10^6 *E. coli* cells demanded 0.06- mg/L ozone after lysis and attributed the second phase of inactivation to this ozone demand.

Relative Ozone Resistance:

Batch Reactor. In batch reactions, *E. coli* O157:H7 was more resistant to ozone inactivation, and *L. monocytogenes* was less resistant than other tested microorganisms (Figure 1.02). Variation in resistance to ozone among microorganisms was reported earlier. Baumann and Ludwig (1962) compared chlorine resistance of different bacteria and viruses and reported that *E. coli* at pH 7 is the most sensitive and *Bacillus anthracis* is the most resistant. Zhao and Cranston (1995) observed a 5-log decrease for *Staphylococcus aureus*, *B. cereus*, *E. coli*, and *Salmonella* in 10 to 20 min when they were sparged in the water with 6.7 mg/L ozone at the flow rate of 6 L/min. They also reported that *S. aureus* and *B. cereus* exhibited greater tolerance to ozone than did *E. coli* and *Salmonella* spp.

Inactivation data were fitted to a dose-response model having two segments (Figure 1.04). According to Finch et al. (1988), the log-log dose-response model, normally used to describe ozone disinfection of natural waters, was inadequate over a range of ozone doses and bacteria concentrations used in their study. Masschelein (1982) and Hoigné (1982) also noted that ozone reaction with microorganisms is dependent upon the concentrations only in the limited range of ozone dose.

Continuous Reactors: Continuous reaction systems were studied to ensure exposing cells to constant ozone concentration during the treatment and to allow measuring fast inactivation rates. In reactor I, streams of cell suspension and ozonated water were mixed continuously in a y-shaped glass tube, but ozone was quickly depleted during the holding period (Figure 1.05). Although this design maximizes bactericidal action of ozone and minimizes ozone depletion by intracellular components, data were only marginally better in describing inactivation kinetics than were the data from the batch system (Figure 1.05).

The reaction system was modified to permit continuous exposure of bacteria to constant concentration of ozone during the treatment. Inactivation data were better described by first-order kinetics when using reactor II than the previously tested systems (Figure 1.06). Survivors plots were linear initially, with a concave downward overall pattern. Inactivation data from all tested bacteria were fitted adequately to a log-log dose-response model (Figure 1.07).

Joret et al. (1982) reported inactivation of 1.5 and 3-logs *E. coli*, present in wastewater, by 1.4 and 2.2 mg ozone/L, respectively, for a 19-min contact time in a continuous-type reactor. Residual ozone concentrations were 0 and 0.06 mg/L, accordingly. These authors found no linear relation between bacterial inactivation rate and contact time. Restaino et al. (1995) evaluated the antimicrobial effects of ozone (1.88 mg/L) in a recirculating concurrent reactor against gram-negative (*S. typhimurium*, *E. coli*, *P. aeruginosa*, and *Yersinia enterocolitica*) and gram-positive (*L. monocytogenes*, *S. aureus*, and *Enterococcus faecalis*) food-borne bacteria. Populations of gram-negative bacteria, except *P. aeruginosa*, decreased > 5-logs instantaneously. In the case of *L. monocytogenes*, the count decreased > 5-logs immediately, but only a decrease

of 3-logs was observed for *S. aureus*, *E. faecalis*, and *B. cereus*. Most bacteria showed biphasic death curves. Restaino et al. (1995) concluded that the gram-negative bacteria were substantially more sensitive to ozonated water than the gram-positive bacteria, which is inconsistent with the findings of this study.

Hunt and Mariñas (1997) investigated the kinetics of *E. coli* inactivation with ozone using semi-batch and continuous-flow tubular reactors in phosphate buffer. Inactivation kinetics were consistent with a pseudo-first -order rate for the first 5 to 7 log units of inactivation. They related the tailing of survivor plots to the presence of bacterial clumps in the reaction mixture. In the experiments performed with the tubular reactors, Hunt and Mariñas (1997) found that the concentration of dissolved ozone decreased as it reacted with the treated microorganisms. Their data showed two phases of kinetics. In addition, from their inactivation study with or without a radical scavenger, *tert.*-butanol, they concluded that molecular ozone rather than free radicals was primarily responsible for inactivation in the range of experimental conditions examined.

Scott and Lesher (1963) assumed that the reaction rate is a function of the frequency of the collisions between the bacterium and the ozone molecules, therefore, the utilized ozone should be correlated with the number of bacteria removed. However, in actual experiments, first-order kinetics are often not observed throughout the entire range of experimental conditions, but rather during only a portion of the experiment (Hoff 1986). Thus survival curves may depart from the ideal exponential kinetics and follow (a) convex downward pattern, which shows an initial lag period before first-order inactivation, (b) concave downward kinetics, that is, a rapid initial decline in populations, or (c) multiple kinetics sometimes referred to as "tailing off". Dahi (1977) observed that sonication before ozonation removed the tailing effect.

Ozone disinfection had two distinct stages: an initial rapid decline in the first stage followed by a slower decline in the second stage (Finch et al., 1988; Katzenelson et al., 1974). Cellular debris from the damaged or lysed organisms shielded the surviving *E. coli* from the effects of ozone (Finch et al., 1988). These authors calculated that 3×10^8 molecules of ozone were used for each bacterium, however, a 7 log unit reduction in *E. coli* required 45 times more ozone than the predicted value. Consequently, Finch et al. (1988) found a dose-response relationship that has a “tail”.

Various explanations for the tailing during disinfection with ozone have been reported. Hoigné (1982) suggested a shielding that results from faster competing reactions for ozone, compared with the disinfection reaction. Consequently, as the ozone dose increases and the concentration of surviving bacteria decreases, cell lysis may occur. Products of cell lysis may compete with living cells for available ozone, thereby shielding remaining viable organisms. It is still unclear whether this shielding varies among microorganisms.

Conclusions:

Ozone inactivates bacteria rapidly, and no viable cells are detectable when residual ozone is present in the reaction mixture. Ozone exerts its action within a few seconds, and therefore inactivation kinetics are not measurable in the batch and the continuous reactor (type I) modes. A continuous ozone reactor II was developed to ensure a uniform exposure of bacterial cells to ozone and constant concentration of ozone during the treatment. When using this system,

inactivation kinetics that are consistent with general disinfection patterns were observed. Therefore, inactivation of microorganisms in a fluid system by aqueous ozone follows patterns that depend on the method of application. Resistance of tested bacteria to ozone followed this descending order: *E. coli* O157:H7, *P. fluorescens*, *L. mesenteroides*, and *L. monocytogenes*.

1.2.7.2.1 As a Sanitizing Agent for Meat Processing Equipment

In October 1999, the Plumrose USA, Inc. ham, turkey, chicken and deli meat processing plant in Booneville, MS replaced its chlorinated detergent and chlorine-based sanitizers with an ozonated water system (EPRI, 1999). Table 1.06 presents the equal or better sanitation levels obtained before and after the processing change was made.

Many additional examples of the antimicrobial effects of ozone will be presented in subsequent sections of this petition that deal with specific types of foods.

1.2.7.3 In Air

In contrast to the extensive literature documenting the antimicrobial activity of ozone in aqueous media, literature documenting ozone's antimicrobial activity in the gas phase is relatively sparse.

Utilization of ozone for increasing the storage life of food, particularly if held at low temperatures, is believed to have started in 1909 when, in the cold-storage plant of Cologne, the reduction in the germ count on the surface of meat stored there was observed after an ozone generator had been installed in the duct of fresh air used to ventilate the storage room (Horváth et al., 1985). Much more extensive examinations and experiments were required on the storage of fruits in cold-storage plants in order to decide whether treatment by ozone could be deemed favorable or unfavorable because of the different requirements imposed on the storage of various fruits. Although few publications or research reports have as yet become part of the public domain, "the use of ozone is increasing in several major cold-storage plants in Europe" (Horváth et al., 1985).

Table 1.06. Comparison of Chlorine vs Ozone Sanitation (EPRI, 1999)

Racks Sampling	Number of Checks	% With Colonies	% With No Colonies
4th Qtr 1998	216	15	93
1st Qtr 1999	188	31	84
2nd Qtr 1999	196	14	93
3rd Qtr 1999	208	13	94
Ozone System Installed October 6, 1999			
Week 1	20	1	95
Week 2	50	3	94
Week 3	50	2	96

Ewell (1938) stated that depending on the cleanliness, minimum continuous concentrations of 0.6 to 1.5 ppm ozone (in air) were necessary to prevent mold growth on eggs kept at 0.6EC and 90% relative humidity (RH), whereas 2.5 to 3.0 ppm ozone were required to control molds on beef that was stored under similar conditions.

Yeasts vary in sensitivity to ozone. Naitoh (1992) treated *Hansenula anomala*, *Saccharomyces rosei*, *Pichia farinosa*, *C. parapsilosis*, *Kluyveromyces marxianus*, and *Debaryomyces hansenii* var. *hansenii* with gaseous ozone at 4 to 5 ppm for 1 to 5 h at 30 to 60EC and 25 to 90% RH. At lower temperature and 5 h exposure, counts of *C. parapsilosis* and *K. marxianus* decreased more than 1-log; however, counts of the other yeasts did not decrease appreciably. The antimicrobial effect increased with increasing temperature, RH, and treatment time. Ozone increased lag and exponential phases of *H. anomala* and *K. marxianus* by 1.5 to 4 and 1.4 to 6.7 h, respectively

Elford and Ende (1942) used low ozone concentrations and long exposures at variable relative humidity to disinfect airborne microorganisms. At RH <45%, the germicidal power of ozone was negligible. Inactivation was substantial even at concentrations far below 0. 1 mg/liter when high humidity was used. Ewell (1946) demonstrated that microorganisms were killed more readily by ozone in an atmosphere having a high rather than low RH.

Practical operations for preservation start with the sterilization of air in such a way that air entering the storage room contains a sufficient amount of ozone to destroy microorganisms (Horváth et al., 1985). At the same time, however, ozone decomposition to a significant extent is to be expected due to the high moisture content required, the walls of the storage room, the packaging materials, the adsorption effect of the stored goods, and also to the oxidation reactions taking place. These two requirements demand the most perfect distribution of ozonized air in the storage room and make it imperative that the capacity of the ozone generator ensures the maintenance of the appropriate ozone concentration throughout the whole mass of air. Otherwise it may happen that ozone will not reach the storage space proper, let alone the surface of the goods stored. The required effect can be attained by a strong air movement; the storage space, in turn, need not be hermetically sealed as, for example, in the case of storage under static CO_2 gas atmosphere. A state of equilibrium can set in, even in these relatively closed premises. between the amount of ozone consumed by the environment, the packaging materials and the walls, etc., through adsorption, and utilized by the stored goods (for the destruction of surface

germs, the oxidation of metabolic products, etc.) on the one hand. and the amount of ozone introduced on the other. The process for attaining the equilibrium is shown in Figure 1.08.

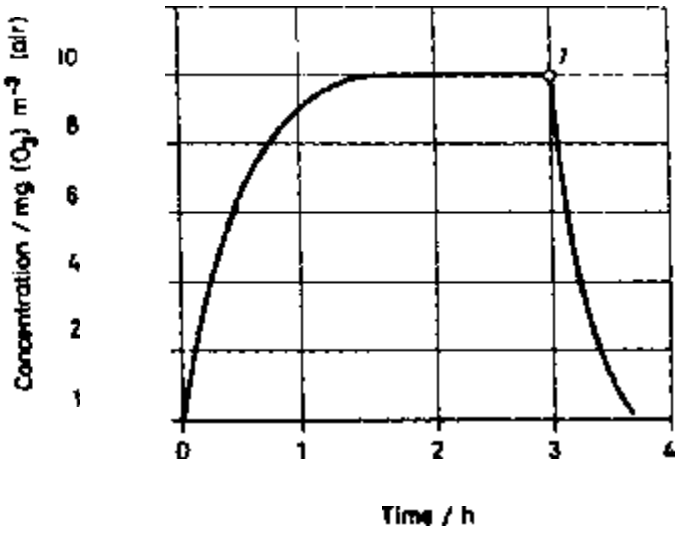


Figure 1.08. Formation of ozone balance in closed experimental space as a function of time at constant ozone feed rate. (1 = stoppage of ozone feed) (Horváth et al., 1985).

After stopping feed, decomposition continues for which ozone is supplied up to a certain time by desorption from the environment; even so, the total depletion of the ozone content sets in rapidly. The concentration of ozone in storage rooms can be calculated according to Ewell ([1938, 1940, 1946, 1950) and see Kuprianoff [1953]) by using the formula:

$$C = \frac{m}{k} = \frac{m}{0.7 / Z_0} mg(O_3)m^{-3}(air)$$

where m is the amount of ozone introduced to the room in a minute, in mg m^{-3}; Z_0 is the half-life of ozone decomposition after stopping the ozone feed, in minutes.

Practically attainable values for Z are between 20 and 50 min. Since the equilibrium sets in rapidly, after about 20 min, even in the case of an intermittent ozone feed, it is advisable to start with the generation of ozone once in every hour as the germicidal effect hardly subsides at all in practice. During storage, ozone exerts a threefold effect by destroying the microorganisms, oxidizing the odors and affecting the processes of metabolism.

The germicidal power of ozone generally is specific with respect to individual species; experience has shown it to be more efficient for molds than for bacteria [Kuprianoff, 1953). It was established that ozone present in low concentrations (about 0.2 mg m^{-3}) is not very efficient against bacteria, because they develop tolerance after a certain time of exposure. Similar behavior is exhibited by certain species of molds found on fruits. Ozone's primary action on molds is to suppress their growth and this effect can set in rapidly, particularly in the initial stage on a mold-free surface. Afterwards, this process leads to the destruction of the cultures already formed. Ozone attacks immediately the easily accessible cells on the surface since ozone exerts a surface effect in the first place and has only a slight depth of penetration.

Kolodyaznaya and Suponina (1975) investigated the microflora causing the deterioration of potato. Pure mold cultures of *Fusarium solani, Rhizoctonia solani*, and *Phytophtora solani* were exposed to the action of ozone. From these species, *Fusarium solani* proved to be resistant to ozone. Ozonization applied for the storage of refrigerated meat destroys surface microorganisms, particularly the family of *Pseudomonas* responsible for spoilage.

Increasing the moisture content of the environment favorably influences the gerrnicidal effect. This is brought about by the swelling of microbes making them more susceptible to destruction, Experiments conducted with beef showed that ozone is most efficicient if the surface has a definite moisture content of around 60 percent (Horváth et al., 1985).

Holah et al. (1995) constructed a special aerobiology cabinet to study the destruction of airborne microorganisms by disinfectant fogging, by ozone and by ultraviolet light. Survival was assessed using a variety of methods including settle plates, precipitation onto metal strips, impaction onto agar (SAS sampler) and impingement in a glass cyclone sampler.

Exposure to ozone above 4 ppm in air (5 and 10 min exposure) significantly reduced the numbers of airborne *P. aeroginosa*. Cyclone results indicated that 2-4-log reductions were possible in 5-10 minutes of ozone exposure in combination with the volume of the aerobiology chamber (0.36 m^3 – 60 cm wide x 60 cm deep x 100 cm in length).

Kowalski et al. (1998) studied the bactericidal effects of airborne ozone against *E. coli* and *Staphylococcus aureus* in a 72-L ozone chamber. Petri dishes containing the microorganisms were placed in the chamber and exposed for 10 to 480 seconds to ozone concentrations between 300 and 1500 ppm. Death rates in excess of 99.99% were achieved for both species (Figures 1.09a and 1.09b).

Table 1.07 lists data on ozonation of airborne bacteria and viruses reported by previous investigators and compiled by Kowalski et al. (1998).

Table 1.07. Ozonation of Bacteria and Viruses in Air (Kowalski et al., 1998)

Test Organism	Ozone (ppm)	Time (sec)	% Survival	Reference
S. salivarius	0.6	600	2	Elford & van de Eude, 1942
S. epidermis	0.60	240	0.6	Heindel et al., 1993
pX174 (virus)	0.04	480	0.1	de Mik, 1977

1.2.8 Safety Aspects of Ozone

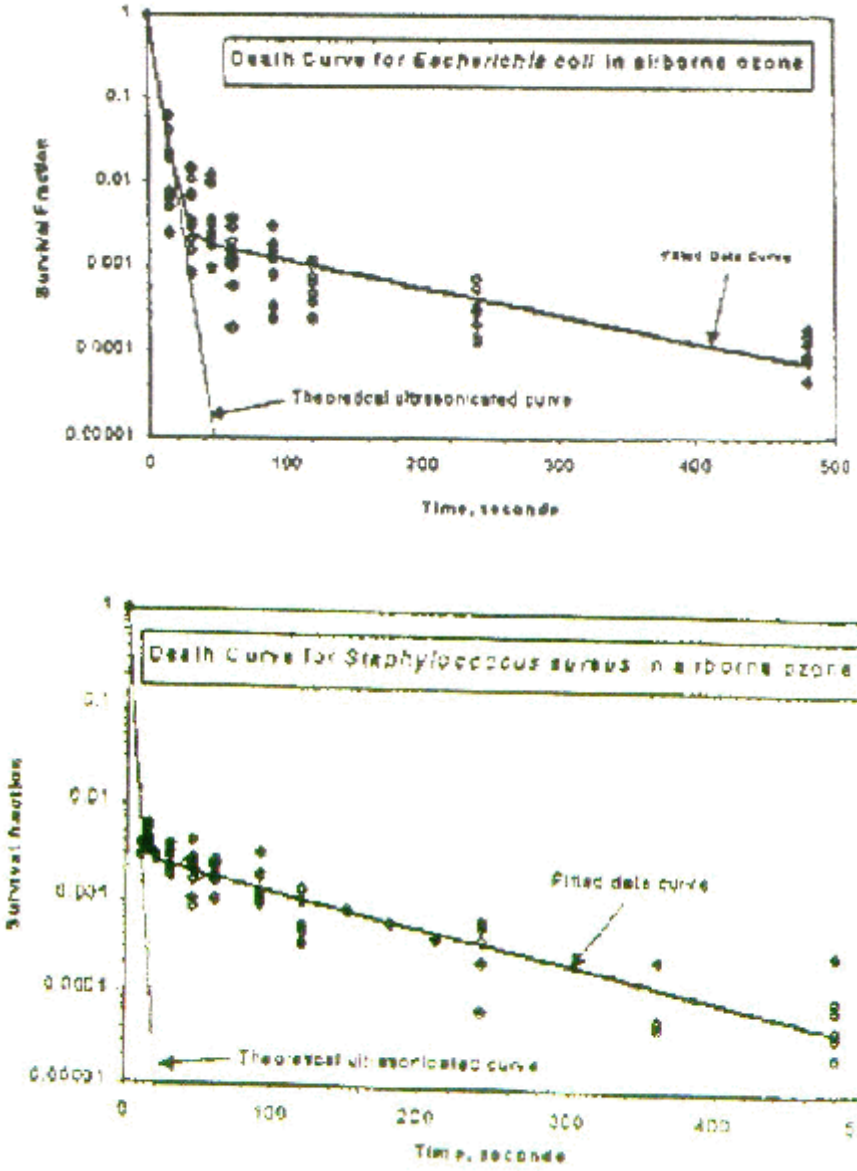
Human Exposure to Ozone. (Wojtowicz, 1996) The toxicity of ozone is largely related to its powerful oxidizing properties. The odor threshold of ozone varies among individuals but most people can detect 0.01 ppm in air, which is well below the limit for general comfort. OSHA has established a time-weighted average permissible exposure level for workers for an eight-hour day of 0.10 ppm v/v (0.2 mg/mL) and a short-term exposure limit of 0.30 ppm v/v (0.6 mg/m^3) for an exposure less than 15 minutes (OSHA, 1975). The latter is based on observations showing that significant declines in pulmonary function can result from repeated intermittent exposures or from a single short-term exposure to ozone. The toxicity of gaseous ozone varies with

concentration and exposure time (Langerwerf, 1963). The symptoms experienced on exposure to 0.1-1 ppm ozone are headache, throat dryness, irritation of the respiratory passages, and burning of the eyes caused by the formation of aldehydes and peroxyacyl nitrates. Exposure to 1-100 ppm ozone can cause asthma-like symptoms such as tiredness and lack of appetite. Short-term exposure to higher concentrations can cause throat irritations, hemorrhaging, and pulmonary edema. Additional toxicity data is given in Lewis (1993).

Because ozone is such a strong oxidation agent, it is not good operating practice to design or operate ozonation systems which allow for humans to be exposed to ozone. Excess ozone in off-gases from ozone contactors should be destroyed prior to discharge to the ambient environment.

1.2.8.1 Exposure Threshold Limit Standards for Ozone (Pryor and Rice, 2000)

The current Permissible Exposure Level - Time Weighted Average (PEL-TWA) for ozone exposure in the workplace environment is 0.1 ppm as recommended by the American Conference of Governmental Industrial Hygienists (ACGIH, 1986) and adopted by the U.S. Occupational Safety and Health Administration (OSHA). This is the concentration to which healthy, susceptible individuals can be exposed continuously to ozone during a normal 8-hour day / 40 hour workweek without adverse effects. The current Permissible Exposure Limit - Short Term Exposure Limit (PEL-STEL) is 0.3 ppm. This is the level to which healthy, non-susceptible individuals can be exposed for a short period of time without suffering from irritation or other acute effects, provided that the PEL-TWA is not exceeded. A PEL-STEL is defined as a 15-minute exposure to ozone that should not be exceeded at any time during the workday even if the 8-hour time weighted average does not exceed the PEL-TWA level. Exposures at the PEL-STEL should not be longer than 15 minutes and should not be repeated more than 4 times per day. There should be at least 1 hour between successive exposures at the PEL-STEL.



Figures 1.09a and 1.09b. Death curves for *E. coli* and *Staphylococcus aureus*, respectively, in ozonated air. Ozone concentrations varied from 300 to 1500 ppm (Kowalski et al., 1998).

1.2.8.2 Toxicology of Ozone

The acute and chronic effects of excessive exposure to ozone have been well investigated (Stockinger, 1965). Exposures to concentrations of ozone in excess of several tenths of a ppm (0- 0.3 ppm) sometimes cause discomfort in a small susceptible portion of the population. This can be in the form of headaches or dryness of the throat and mucous membranes of the eyes and nose following exposures of short duration (Wilska, 1957; Truche, 1951). Repeated exposure to

ozone at such concentrations at 24-hour intervals, however, causes no further increase in airway irritability. In fact, some research has demonstrated that additional exposures to ozone beyond the first exposure had progressively lesser effects, suggesting that tolerance may develop to these effects of ozone exposure (Nadel, 1979).

Ozone has been shown to be more injurious at concentrations exceeding 2.0 ppm over several hours (Stockinger, 1951), such as experienced by gas-shielded arc welders. The primary site of acute effects is the lung which is characterized by pulmonary congestion. This acute impact subsided in welders when exposures were reduced to less than 0.2 ppm (Challen et al., 1958). Based on animal studies, exposures over 10-20 ppm for an hour or more are believed to be lethal in humans, although there has never been a single recorded fatality attributed to ozone overexposure during the more than 100 year history of its commercial use.

With respect to long term or chronic toxicity, ozone is a radiomimetic agent. That is, prolonged exposure to excessive ozone levels exhibits the same effects to humans as does excessive exposure to sunlight. These effects include drying of the dermal surfaces and general aging of exposed tissue. According to the ACGIH (1986), ozone is neither a confirmed nor suspected human carcinogen. Nor does it exhibit teratogenic or mutagenic properties.

1.2.8.3 Comparative Safety Aspects

In many industrial applications, ozone is the gaseous oxidant of choice. This is due to the following properties of ozone or its manufacture (Pryor, 1990):

1. Upon Human Exposure, Ozone Exerts Only Temporary, Acute Symptoms of Exposure – Except in very rare cases of extended, severe overexposure to high concentrations of ozone (several hours at greater than 2-3 ppm), the physical symptoms of ozone exposure are acute and transitory in nature (Stockinger, 1965). These symptoms include watery eyes, tightness in the chest, shortness of breath, and irritated throat. Headaches or light-headedness are common. Recommended treatment of excessive exposure to ozone includes removal of the exposed personnel from the area of exposure and rest, except in severe cases of overexposure for which oxygen is recommended for inhalation by the patient. Symptoms generally begin to subside within minutes once the exposure is ended, and complete recovery occurs within hours or, in the most severe exposure cases, days. Ozone is not characterized as a carcinogen, a mutagen, or a teratogen. Ozone also does not accumulate in body fatty tissue nor does it generally cause long-term chronic effects to humans.

2. Ozone has the Highest Oxidation Potential of Commercially Available Oxidizing Agents -- Any chemical with a high oxidation potential has faster reaction kinetics than other materials. This means that either less of the chemical or reduced contact times are required to obtain the desired oxidation reactions compared to those of weaker oxidizing agents.

3. Ozone is Manufactured On-Site, at Relatively Low Concentrations and Pressures (20 psi or below, usually below 15 psi) -- It is immediately consumed in the treatment process. Because of its inherent instability, ozone cannot be stored as a compressed gas. An uncontrolled, widespread, and immediate release of large quantities of ozone thus is not possible to the extent

that the sudden releases of the entire contents of containers of other bulk-stored, concentrated chemicals can occur in the event of an industrial accident or natural disaster.

4. Ozone Has a Comparatively Short Half-Life -- Ozone's half-life generally is measured in minutes in the aqueous phase to hours in the gas phase. Any accidental releases of ozone will not persist in the environment as long compared to the more stable oxidizing agents under the same conditions. Nevertheless, all systems designed to produce and apply ozone should include appropriate ozone-destruction units to prevent the possibility of ozone being present in plant atmospheres.

5. Ozone Decomposes Into Simple Diatomic Oxygen Upon Breakdown - It will not form environmentally harmful or persistent compounds upon reaction with common hydrocarbons -- nor will it form chlorinated hydrocarbons such as chloroform.

6. Ozone Has a Characteristically Strong Odor -- Ozone can be detected by human olfactory senses at concentrations as low as 0.01; or one-tenth of the OSHA-allowable PEL-TWA. Thus it is readily detected by an individual at concentrations well below harmful concentrations. Further, the pungent smell is so powerful at concentrations above 0.5 ppm that normal individuals will quickly remove themselves from such an excessive exposure well before significant exposures are recorded unless incapacitated or exits or egress are otherwise blocked.

Despite these properties, safe practice in plants of any type using ozone is to install ambient air ozone monitors to sense continually for ozone levels. Such monitors can be pre-set to flash lights, sound an alarm, and even start up exhaust fans if the monitor detects ozone at levels above the set-point (which in many plants is just below the OSHA PEL of 0.1 ppm.

7. Ozone is Considered to be Freely Dispersed in the Atmosphere According to U.S. Environmental Protection Agency (EPA) Models -- Although this is a true statement for atmospheric ozone models which include wind effects, it is important to recognize that ozone gas is slightly heavier than air. Consequently, if ozone is discharged to a work-place atmosphere or exhausted from a building by fans, it can slowly settle to ground level on still days. Once at ground level, ozone can slowly decolorize local vegetation and attack easily oxidizable materials on automobiles (esp. rubber tires and windshield wipers).

1.3 REGULATORY CONSIDERATIONS

Use of ozone in food processing currently is regulated by a 1982 GRAS Ruling on the use of ozone in Bottled Water, issued under 184.1 (b) (2) which required any other uses to be regulated by food additive petition. Ozone at that time was evaluated only for use in Bottled Water, the ruling was unduly restrictive, and should have been issued under 184.1 (b) (3) permitting other uses to be GRAS pending adequate evaluation. An Expert Panel performed such comprehensive review and evaluation in 1997, resulting in a declaration of GRAS Status for broad use of ozone in food processing (EPRI, 1997; Graham, 1997). In the light of current knowledge, we believe it is prudent to modify the 184.1 (b) (2) ruling and reissue it under 184.1 (b) (3), permitting GRAS Affirmation of the use of ozone as an Antimicrobial Agent in Food Processing.

1.4 SPECIFICATIONS ON USE LEVELS AND BYPRODUCTS

Some examples of effective application of ozone in air or in water, based on data presented in the following sections of this petition, are listed in Table 1.08. The data are presented as examples only and are not to be construed as limiting control standards. Ozone generally is effective against microorganisms associated with foods, however the optimum use levels are not identical for all foods. Because ozone degrades rapidly, especially in the presence of ozone-demanding materials such as food surfaces, continuous application is necessary to produce the minimum level of ozone required for the intended Antimicrobial Effect(s).

A persistent residual level of ozone is not found in finished food products. Application levels are self-limited by the low solubility of ozone in water, and are further constrained by the EPA prohibition against atmospheric release of ozone and the established OSHA limits on ozone in the workplace environment. In Table 1.08, the term "Minimum Treatment Level" refers to the constant ozone level maintained in contact with the target product for the indicated treatment time.

Oxidation byproducts resulting from ozone use as an Antimicrobial Agent are normal food oxidation products, which pose no reported toxicity or carcinogenicity. This subject has been discussed in depth in section 1.2.3.

Table 1.08. Examples of Effective Continuous Ozone Treatment Conditions for Some Selected Individual Foods and Target Applications

Food Type And Application	Continuous Ozone Exposure Conditions			Comments and/or References
	Minimum Residual Level in		Minimum Treatment Time, minutes	
	Air mg/m^3 (ppm)	Water mg/L (ppm)		
Poultry carcasses		6	30	EPRI, 1999b
Salmonella on chicken carcasses		0.35	30	Caracciolo, 1990 – 40 MPN/g Salm --> <3. 930 *Proteus* --> 9
Poultry chiller water		3.0-4.5	45	Sheldon & Brown, 1986a; 78, 91, 81% redns of APC, coliforms and *Salmonella*
Spent broiler neck chiller water		3.0-4.5	15	Sheldon & Chang, 1987a; 99.5, 99.52, 99.5, 99.9% redns of APC, coliforms, *E. coli*, and *Salmonella*
Poultry chiller water		3-7	15-30 – BOC Macron™ Loop	Ark. Ag. Exptl. Sta., 1997; > 90% redns of APC, *E. coli*, and coliforms
Poultry hatchery – air disinfectant	1.51-1.65% by weight		8	Whistler & Sheldon, 1989b; >4-7-logs redn in bacteria & fungi
Chicken Broiler Parts		3.8	20	Yang & Chen, 1979b; 85-90% count reduction
Beef muscle slices	0.6		constant @ 0.3EC	Kaess & Weidemann, 1968
Fresh mackerel, gutted and washed		0.6	30	Haraguchi et al., 1969; Washing repeated E.O.D.
Vibrio control in shrimp mariculture		0.07-0.08 (seawater)	3-6 hrs	Blogoslawski et al., 1993
Apples in Storage	1-2; 85-90% RH		1 hour/day	Smock & Watson, 1942 lowers/eliminates molds

Food Type And Application	Continuous Ozone Exposure Conditions			Comments and/or References
	Minimum Residual Level in		Minimum Treatment Time, minutes	
	Air mg/m^3 (ppm)	Water mg/L (ppm)		
Molds on Newtown apples stored 3 mos.	1-2		3 months	Smock & van Doren, 1939; no mold growth; no scald
Spores (*P. expansum; S. fructicola*) on appples	0.6; 85-90% RH		3-4 hrs	Smock & Watson, 1942
Fruit storage – general conditions	1-3; >90% RH		2-3 hrs/day	Kuprianoff, 1953
Thornless blackberries storage	0.1-0.3 @ 2EC		12 days	Barth et al., 1995; O_3 suppressed fungal growth 12 days
Grapes storage	0.1		20-40	Sarig et al., 1996; O_3 reduced fungal berry decay
Strawberries		2.7	0.5	Lyons-Magnus, 1999; Reduced *E. coli*, SPC
Chinese cabbage washing		2-3	< 60	Kondo et al., 1989; > 90% redn in total bacterial counts
Broccoli washing Carrots washing Broccoflower washing		1.1 0.64 1.08	10 10 10	Hampson et al, 1994; 3-log redn of APC 2-log redn of APC 1-2 log redn of APC
Carrots storage	15 ΦL/L air		8 h/day @ 2EC	Liew & Prange, 1994; gave some disease protection with minimal change (lighter color)
Broccoli florets washing		1	10-50, then 4 days storage	Zhuang et al., 1996; microbial growth inhibited
Broccoli washing Broccoflower washing Carrots washing		1 1 1	6.0/1-log 7.5/1-log 9.6/1-log	Hampson/ Fiori, 1997; for higher log-enacts, increase $[O_3]$ or contact time with wash water

Food Type And Application	Continuous Ozone Exposure Conditions			Comments and/or References
	Minimum Residual Level in		Minimum Treatment Time, minutes	
	Air mg/m^3 (ppm)	Water mg/L (ppm)		
Lettuce		1.3	3	Kim et al., 1999; 3- to 4-log count reduction
Whole Grains	50		120	Naito et al., 1987a
Japanese raw noodles	0.5 ~ 50		6 hr	Naito et al., 1989c; increases storage life 2-5 times
Confectionery plant air	0.03-0.112 O_3 generated by UV bulbs		10 h/day at night (no workers)	Naitoh, 1989d; lowered airborne bacterial & fungal counts ~ 50%
Ground Black Pepper	6.7		60	Zhao & Cranston, 1995
Bacillus cereus spores		2.5	5	Broadwater et al., 1973
Bacillus cereus vegetative		0.12	5	ditto
E. coli		0.26	1.7	Farooq & Akhlaque, 1983
Salmonella typhimurium		0.26	1.7	ditto
Sanitizing wine processing equipment with water washing		1.5	~ 2	Hampson, 2000; reduces plate counts 63.2-99.9%

2.0 SUPPORTIVE DATA FOR SPECIFIC OZONE APPLICATIONS

Many examples of the antimicrobial action of ozone in direct contact with foods and/or with food microorganisms have been reported in the literature. These have been assembled and categorized into a few relevant applications for ozone, and are discussed below.

2.1 POULTRY

2.1.1 Poultry Meat Studies

2.1.1.1 Yang and Chen (1979a) – Broiler Carcasses

These investigators applied ozone to aqueous suspensions of microorganisms obtained from fresh ground broiler carcasses and from spoiled gizzards.

Testing Procedure: Ground broiler carcasses were diluted with distilled water (1:9) and filtered through Whatman No. 1 filter paper for microbial suspension. Broiler gizzards were stored at 4EC for 10 days to obtain spoiled samples. Ten pieces of spoiled gizzards were washed with 100 mL of distilled water. Twenty mL of this spoiled microbial suspension was diluted with distilled water or test solutions (Ringer solution, NaCl solutions, or egg albumin solutions) to 600 mL final volume.

Ozone from an ozone generator was connected to a 500 mL washing bottle which contained 300 mL of the poultry meat bacterial suspension or spoiled gizzard bacterial suspension. The washing bottles were maintained at 25EC or in ice slush. Control samples were treated with compressed air or oxygen under the same flow conditions as for the ozone treatments. All studies were repeated two times.

Total microbial counts were made immediately following treatment. One mL of the treated suspension was removed and serial dilutions were made using nutrient broth. Plate counts were conducted in duplicate employing standard method agar (BBL). Plates were incubated 72 h at 20EC.

Results: Treatment of spoiled poultry meat microbial suspension at 25EC at a log count of 7.08/mL with ozone at 19 mg/L for 4 min under a gas flow rate of 3175 mL/min completely destroyed microorganisms (Figure 2.1.01). At 2EC, fresh poultry meat microbial suspension with an initial log count of 3.63/mL was completely destroyed by ozone treatment at 37.7 mg/L for 10 min under a flow rate of 650 mL/min. Under the same conditions, a lesser bactericidal effect was observed at 25EC (Figure 2.1.02). Higher ozone concentrations and longer ozone contact time were needed for fresh country meat microorganism destruction than for spoiled poultry meat microorganism destruction.

In liquid phase, spoiled poultry meat microorganism destruction by ozone was affected by pH value (Figure 2.1.03). At 2EC and pH values of 3, 5, 7, 9 and 11, ozone treatment at 2.48 mg/L for 5 min under a flow rate of 3175 mL/min resulted in 4.74, 3.25, 3.17, 3.22 and 1.25 log cycle reduction in microbial counts. No differences were apparent in the percentage of microbial reduction at pH values of 5, 7 and 9. Results clearly indicated that at lower pH values, such as

3.0, higher ozone bactericidal effects were found, while higher pH values, such as 11.0, had a lower bactericidal effect. This behavior can be explained on the basis of known ozone chemistry. At the lower pH values, molecular ozone is the major species present. However, at pH 11, decomposition of ozone to the unstable hydroxyl free radical proceeds rapidly. And since the half-life of hydroxyl free radical is on the order of microseconds and its concentration in water is never greater than 10^{-12} M, little antimicrobial activity can be expected from it.

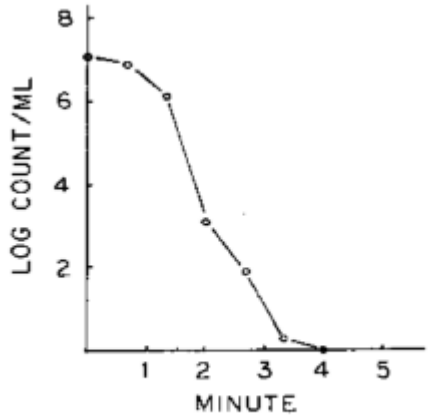


Figure 2.1.01. Reduction of total microbial counts by ozone treatment. Microbial suspensions from spoiled poultry meat at 25EC were treated with ozone at 19 mg/L under a flow rate of 3175 mL/min (Yang and Chen 1979a).

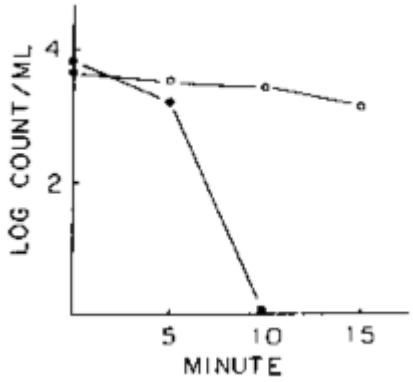


Figure 2.1.02. Reduction of total microbial counts by ozone treatment as affected by solution temperature. Microbial suspensions from spoiled poultry meat at 25EC were treated with ozone at 37.7 mg/L under a flow rate of 650 mL/min. " = 25E; ! = 2E (Yang and Chen 1979a).

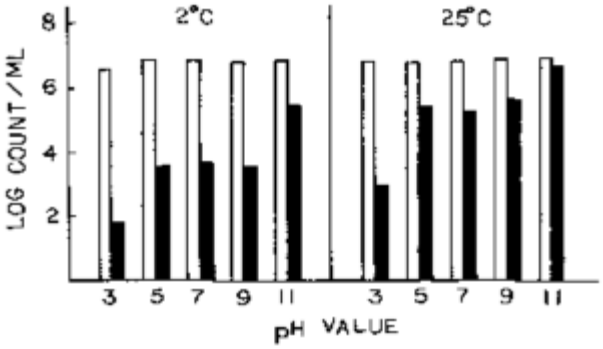


Figure 2.1.03. Effects of pH on the reduction of total microbial counts by ozone treatment. Microbial suspensions from spoiled poultry meat at different pH values were treated with ozone at 2.48 mg/L for 5 min under a flow rate of 3175 mL/min. 9 = control sample; = ozone-treated sample (Yang and Chen 1979).

At 25EC with pH values of 3, 5, 7, 9 and 11, approximately 3.78, 1.41, 1.68, 1.25 and 0.30 log cycles of the microorganisms were destroyed. Results also indicated that, regardless of the pH value, the bactericidal effect of ozone was much greater at 2EC than that at 25EC. At 2EC and at pH values of 3, 5, 7, 9 and 11, ozonation of water with 45.66 mg O_3/mL for one min under a flow rate of 2050 mL/min resulted in 4.23, 3.19, 3.78, 2.60 and 0 mg/mL residual ozone, accordingly. The authors assumed that the destruction of poultry meat microorganisms by ozone at different pH values was affected by the ozone solubility, but the more rapid rate of ozone decomposition as pH increases also is important. Ozone solubility in water increases as temperature is lowered – at the same time, the decay rate of ozone decreases as temperature is lowered.

Microorganism destruction by ozone was less effective in Ringer solution (a solution of sodium, potassium and calcium chlorides in distilled water) than in distilled water (Figure 2.1.04); possibly, the presence of some Ringer solution ingredients protected microorganisms against ozone or reacted with ozone.

Microorganism destruction by ozone was affected by the presence of sodium chloride (Figure 2.1.05). Treatment of microbial suspensions, containing 0, 1.0, 2.5 and 5.0% NaCl, with ozone at 2.48 mg/L for 5 min under a flow rate of 3175 mL/min showed reductions of 3.57, 4.59, 2.34 and 3.69 log cycles in counts. Repeated studies have indicated that a NaCl concentration below 2.5% enhanced the bactericidal effect of ozone, while 5% NaCl showed a slight protective effect. Ozonation of NaCl solutions with 45.66 mg O_3/mL for one min under a flow rate of 2050 mL/min resulted in 4.62, 4.35 and 3.45 mg O_3/mL for 1.0, 2.5 and 5.0% NaCl solutions, respectively. Results also indicated that the difference in the effect of NaCl concentrations on the destruction of poultry meat microorganisms by ozone was due to the solubility of ozone.

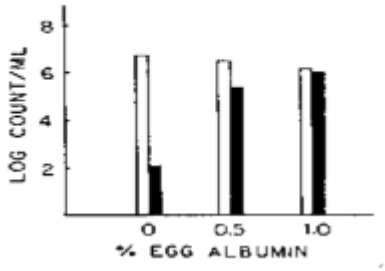


Figure 2.1.04. Protection effect of Ringer solution on the reduction of total microbial counts by ozone treatment. Spoiled poultry meat microorganisms in distilled water and ringer solution at 25EC were treated with ozone at 19 mg/L under a flow rate of 3175 mL/min. " = distilled water; ! = Ringer solution (Yang and Chen, 1979a).

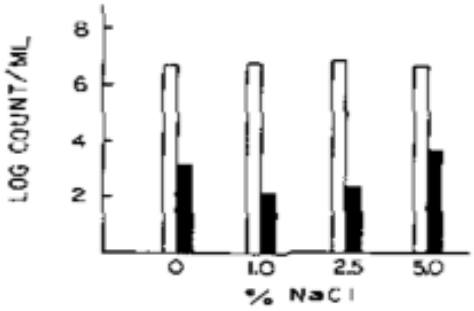


Figure 2.1.05. Reduction of total microbial counts by ozone treatment as affected by the presence of NaCl. Microbial suspensions from spoiled poultry meat at 2EC with various levels of added NaCl were treated with ozone at 2.48 mg/L for 5 min under a flow rate of 3175 mL/min. Q = control sample; O = ozone-treated sample (Yang and Chen, 1979a).

Microorganism destruction by ozone was also affected by albumin (Figure 2.1.06). When microbial solutions containing 0, 0.05 and 1.0% albumin were treated with ozone at 2.48 mg/L for 7 min at a flow rate of 3175 mL/min, about 5.63, 1.06, 0.15 log cycles of the microorganisms were destroyed, respectively. The presence of soluble organic material reduced the ozone bactericidal effect in the liquid phase. This is a logical expectation in light of the strong oxidizing power of ozone, which allows it to react less selectively than weaker oxidizing/disinfecting agents.

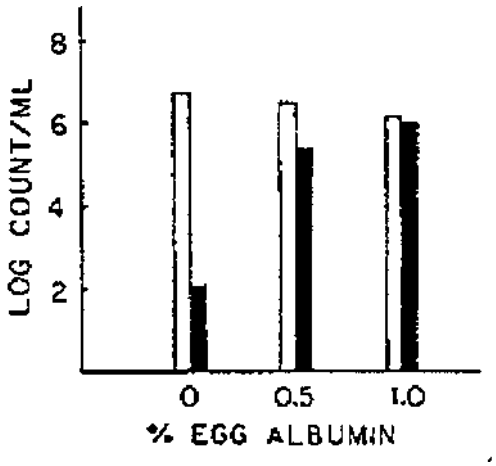


Figure 2.1.06. Reduction of total microbial counts by ozone treatment as affected by the presence of egg albumin. Spoiled poultry meat suspensions with added egg albumin were treated with ozone at 2.48 mg/L for 7 min under a flow rate of 3175 mL/min. Q = control sample; O = ozone-treated sample (Yang and Chen, 1979a).

2.1.1.2 Yang and Chen, 1979b – Broiler Parts

This study showed that ozone treatment of broiler parts effectively reduced levels of gram-negative microorganisms on the products and that ozone treatment does not affect the multiplication of the surviving microorganisms.

Testing Procedures: Broiler Parts: Frozen thigh and breast parts were used in this study. **Poultry Meat Microbial Suspensions:** Chicken neck samples were obtained from a poultry processing plant and stored in a -18EC freezer, Before the study, five pieces of frozen chicken necks were defrosted and washed with 200 mL of distilled water. Sixty mL of the chicken neck microbial suspension was diluted with ice cold distilled water to 600 mL. Chicken necks of the same source were stored at 4EC for 28 days to obtain spoiled chicken neck samples. Three pieces of rotten chicken necks were washed with 200 mL distilled water and 20 mL of this rotten chicken neck microbial suspension were diluted with ice cold distilled water to a final volume of 600 mL. The distilled water used in this study was sterile.

Ozone Treatment: Eight pieces of the cutup broiler parts were weighed and soaked in the same quantity of ice cold water. Using a washing bottle disperser, ozone at 3.88 mg/L under a flow rate of 2050 mL/min was dispersed through the broiler parts for 20 min. Control treatments were made using compressed air under the same flow rate condition as the ozone treatment. The control and ozone-treated broiler parts were drained to remove the excess water and stored at 4 to 5EC in polyethylene poultry bags. For poultry microbial suspension, the ozone outlet was connected to a 500-mL washing bottle, which contained 300 mL of the microbial suspensions.

The washing bottles containing the microbial suspension were maintained in ice slush. The poultry microbial suspensions were treated with ozone at 2.48 mg/L for a predetermined time

under a flow rate of 3175 mL/min. Again, control treatments were made using compressed air under the same flow rate. Each study was repeated three times.

Microbiological Method: Total Count: Microbial samples of the broiler parts were obtained by swabbing the skin for 30 sec in different directions with sterile cotton swabs. An area of 1 in^2 or 6.45 cm^2, described by a sterile aluminum foil template was assayed. Serial dilutions of the swabs were made and plated with standard method agar (BBL). Plates were incubated for 72 h at 20EC to determine the psychrotrophic counts, 48 h at 37EC to determine the mesophilic counts, and 10 days at 7EC to determine the psychrophilic counts. For poultry microbial suspensions, total microbial counts were made immediately following treatment. One mL of the sample was pipetted into serial dilution tubes containing 9 mL of diluent. Mesophilic, psychrotrophic and psychrophilic counts were made as described previously.

Coliform Most Probable Number (MPN): Methods for the coliform test as described by Thatcher and Clark (1968) were followed. Isolation of Colonies: Psychrotrophic count plates were used. Fifty to 60 colonies were randomly removed from the plates containing 10 to 30 colonies; cultures were purified by the streak-plate technique.

Identification of Microorganisms: The purified cultures were transferred to standard method agar slants and incubated for 24 h at 25EC. Fresh cultures were used for the identification. The gram stain, motility, oxidative and fermentative metabolism, cytochrome oxidase, and catalase tests were made according to the methods described by Collins and Lyne (1970). The scheme of Freeman et al. (1976) was used to identify the isolates.

RESULTS AND DISCUSSION

Effect of Ozone on the Microbial Counts of Refrigerated Broiler Parts: The ozone treated broiler parts had consistently lower microbial counts than the control parts during the entire refrigeration period. Immediately after ozone treatment, the total microbial counts at 37, 20 and 7EC were reduced 90.5, 90.5 and 86.0%, accordingly, as compared to the air treated controls. Results indicated that ozone has a slightly stronger destruction power for the mesophiles and the psychrotrophs than for the psychrophiles. The initial 7EC counts were lower than the 37EC and the 20EC counts for both the ozone treated and control parts.

According to Essary et al. (1958), broilers were considered spoiled when the number of psychrotrophs reached a log number of 7.0/cm^2. This log microbial count of 7.0/cm^2 has been accepted as a shelf-life determinating criterion for broiler carcasses (Cox et al. 1974). With this in mind, an extension of 2.4 days in shelf-life was found when broiler parts in ice water were treated with ozone at 3.88 mg/L for 20 min under a flow rate of 2050 mL/min as compared to the air treated control.

Ozone Treatment and Fresh Poultry Meat Microflora: After a fresh microbial suspension was treated with an ozone concentration of 2.48 mg/L for 5 min under a flow rate of 3175 mL/min, its log total count at 20EC was reduced from 2.15 per mL to 1.53 per mL. Also, the log MPN of coliforms was reduced from 1.38 to 0.36. A total of 113 cultures was isolated and purified from the 20E total count plates with 55 cultures from the ozone treated sample and 58 cultures from the control sample. Gram-positive cocci dominated the microbial population on

the ozone-treated sample (Table 2.1.01). Ozone-treated samples contained about 52.7% gram-positive cocci, while the control samples had 39.6% gram-positive cocci. The percentages of gram-positive rods for the ozone-treated samples and the control samples were about the same. The control had 22.4% gram-negative rods, while the ozone treated samples had only 12.7%. It could be postulated that ozone was more effective against gram-negative rod-type organisms than any of the other types.

Table 2.1.01. Incidence of Microflora from Air- and Ozone-Treated Poultry Meat Suspensions [1] (Yang and Chen, 1979b)

Gram-stain and Morphology	Microbial Groups [2]	Air-Treated		Ozone-Treated	
		No. of isolates	% of isolates	No. of isolates	% of isolates
G+ cocci		23	39.6	29	52.7
G+ rod					
	A (*Microbacterium*)	9	15.1	6	10.9
	B (*Lactobacilli*)	6	10.3	12	21.8
	Corynebacterium	7	12.1	1	1.8
G- rod		13	22.4	7	12.7
	C (*P fluorescens* and *P. putida*)	2	3.45	1	1.82
	E (*Pseudomonas sp.*)	3	5.20	2	3.64
	G (*P. putrefaciens*)	2	3.45	—	—
	K (*Acinetobacter*)	2	3.45	1	1.82
	N (*Flavobacterium*)	2	3.45	2	3.64
	-- Others --	2	3.45	1	1.82

[1] Fresh poultry meat suspensions were treated with 2.48 mg/L ozone and air for 5 min under a flow rate of 3175 mL/min.

[2] Microbial groups as described by Freemen et al. (1976) were followed.

Ozone Treatment and Spoilage Poultry Meat Microflora: Ozone treatment drastically reduced the total count at 20EC for spoilage microorganisms. After a spoilage microbial suspension was treated with an ozone concentration of 2.48% mg/L for 9 min at a flow rate of 3175 mL/min, its log total count of 8.30 per mL was reduced to 4.95 per mL and the log MPN of coliforms was reduced from 4.97 per mL to 2.63 per mL.

A total of 119 cultures was obtained with 60 cultures from the ozone treated sample and 59 cultures from the control sample. The gram-negative rod-type organisms dominated and represented 93.2% of the isolates. The results agree with Ayres (1960), and Arafa and Chen (1977), who suggested that the principal microflora responsible for the spoilage of poultry meat were the gram-negative rod-type organisms. After ozone treatment, the number of gram-negative rod-type organisms was reduced and represented 88.3% of the isolates (Table 2.1.02). A slightly higher incidence of gram-positive rods was observed for the ozone treated sample than for the control, 11.7% versus 6.8%. This study has clearly demonstrated that ozone treatment of broiler parts effectively reduced gram-negative microorganisms on the products. This ozone treatment does not affect the multiplication of the surviving microorganisms.

Table 2.1.02. Incidence of Microflora from Air- and Ozone-Treated Spoiled Poultry Meat Suspensions [1] (Yang and Chen, 1979b)

Gram-stain and Morphology	Microbial Groups [2]	Air-Treated		Ozone-Treated	
		No. of isolates	% of isolates	No. of isolates	% of isolates
G+ rod		4	6.8	7	11.7
	A (*Microbacterium*)	4	6.8	6	10.0
	B (*Lactobacilli*)	---	—	1	1.7
G- rod		55	93.2	53	88.3
	E (*Pseudomonas sp.*)	53	89.8	51	85.0
	G (*P. putrefaciens*)	—	—	2	3.3
	– Others --	2	3.4	---	—

[1] Spoiled poultry meat suspensions were treated with 2.48 mg/L ozone and air for 9 min under a flow rate of 3175 mL/min.
[2] Microbial groups as described by Freemen et al. (1976) were followed.

2.1.1.3 Kim and Kim (1991) – Poultry Meat Microorganisms (Information from English abstract, figures and tables)

The utilization of ozone as a disinfectant for removing poultry meat microorganisms and then cleaning the poultry rinse water was investigated. When microbial suspensions were treated with ozone at 2500 ppm/min for 40 min, microorganisms were not well detectable, The bactericidal effect of ozone with temperature was enhanced greater at 7EC than 25EC. All poultry meat microorganisms were killed by ozone treatment at 1530 ppm for 50 min. The pathogenic bacteria such as *Salmonella sp.* were more vulnerable and not detected by ozone treatment for 20 min. Ozonation of the suspension for 20 min and 50 min increased light transmission at 500 nm to 58% and 145%, respectively. The order of COD removal was ozone treatment (21%), coagulant [$Al_2(SO_4)_3$] treatment (41%), ozone treatment after coagulant treatment (64%).

Significant microbial data from this reference are presented in Table 2.1.03 and Figures 2.1.07 and 2.1.08.

Table 2.1.03. Changes in Microbial Flora Level During Ozone Treatment (Kim and Kim, 1991)

Microorganisms	Ozone Treatment Time (min)		
	0	20	50
Aerobic bacteria	4.90 ∀ 0.06 [3]	1.73 ∀ 0.32	ND [4]
Psychrotrophic bacteria	3.83 ∀ 0.40	1.00 ∀ 0.48	ND
E. coli [1]	3.31 ∀ 0.10	0.58 ∀ 0.01	ND
Salmonella sp. [2]	1.89 ∀ 0.29	ND	ND

[1] Most probable number, MPN/mL of rinse water
[2] Most probable number, MPN/100 mL of rinse water
[3] Data represent mean ∀ SD of three experiments
[4] None detected

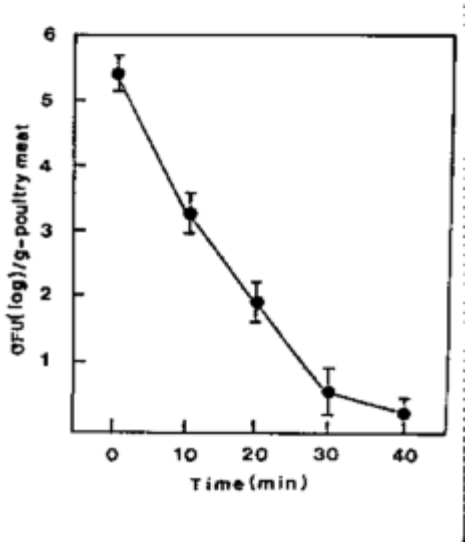


Figure 2.1.07. Reduction of total microbial counts by ozone treatment. Fresh country meat at 7EC was treated with ozone at flow rate of 2500 ppm/min. Each value plotted was the arithmetic mean ∀ SD of 3 experimental replicates (Kim and Kim, 1991).

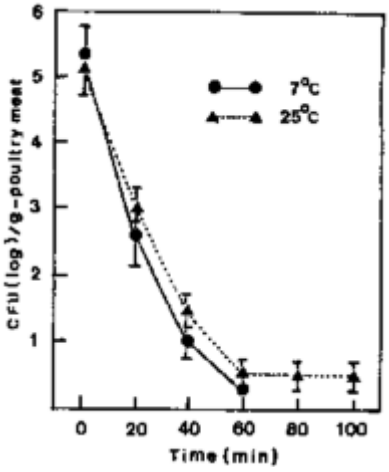


Figure 2.1.08. Effect of ozone treatment temperature on the colony forming unit (CFU, log) of poultry meat. Each value plotted was the arithmetic mean ∀ SD of 3 experimental replicates (Kim and Kim, 1991).

2.1.1.4 Mulder (1995) – Salmonella *Treatment on Poultry Carcasses*

Table 2.1.04 gives the results of reduction of *Salmonella* CFU-counts from an experiment in which poultry carcasses were treated with ozonated water (3.0-4.5 mg/L aqueous ozone concentration). The positive effect of ozone is evident. Ozone treatment also was noted to extend the shelf life of the products and does not result in changes in color or flavor of the product.

Table 2.1.04. *Salmonella* Counts on Poultry Carcasses Treated with Ozone (Mulder, 1995)

	Log *Salmonella* - CFU/g
Control	1.36
Water	1.08
Ozone-Containing Water	0.64

2.1.2 Poultry Chiller Water Treatment Studies

2.1.2.1 Caracciolo (1990) – Turkey Scap Meat

The following data were developed establishing the efficacy of direct contact of ozone for the control of microorganisms on the surfaces of poultry and meat products. These data were included in a Food Additive Petition submitted to the FDA in 1990 by Food Techniques, Inc., Atco, NJ, since withdrawn without prejudice.

Test #1 - Exposure of Turkey Scap Meat to Ozone

The following test was conducted on the premises of a major turkey processing facility in New Jersey on January 15, 1988, using on-line processing equipment. The test was conducted after the plant had been shut down for the day.

Turkey scap meat (1,285 pounds) which had been frozen and thawed twice was loaded into the plant's Challenge Cook Tumbler, and the tumbler was evacuated. As a consequence of the scap meat having been frozen and thawed twice, there was significant bacterial decontamination present (see Table 2.05, Bacterial Analyses, below).

In a first testing procedure, the meat was added to the tumbler along with 257 pounds of water containing 1.33 mg/L of dissolved ozone. The aqueous ozone solution had been prepared by passing ozone plus air through local municipal tap water for a period of approximately four minutes. The concentration of ozone in air exiting the corona discharge ozone generator was approximately 1.12% by weight.

In a second test stage, a 55-gal stainless steel covered drum was filled with the output of the ozone generator containing 1.12% by weight of ozone. This gaseous ozone/air was transferred into the tumbler under suction generated by the vacuum in the tumbler. Air was drawn by vacuum suction from the drum until no more ozone was detected (by its characteristic odor) in the drum (about five minutes, during which time 3-5 volume flushes were obtained). Two drums full (138 gallons) of air containing 1.12% by weight of ozone were so transferred into the tumbler.

In a third test, the output gas from the ozone generator (ozone concentration 1.12% by weight) was fed directly into the tumbler until the volume of ozone-containing gas totaled 9.45 ft^3 (267.6 L).

The first and third procedures were the more convenient and accurate methods of introducing ozone into the tumbler. The total amount of ozone fed into the tumbler for each test is calculated as follows:

1. Ozone dissolved in water:

257 lbs x 454 g/lb x 1.33 x 10^{-6} = <u>0.16 g of ozone in the volume of water charged to the tumbler in the first test.</u>

2. Ozone in the gas from two drum volumes charged to the tumbler:

110 gal. x 0.1337 ft^3/gal x 33.46 g/ft^3 x 0.0112 = 5.51 g of ozone.

3. Ozone in gas charged directly from the ozone generator:

9.45 ft^3 x 33.46 g/ft^3 x 0.0112 = 3.54 g of ozone.

The total weight of ozone charged to the 1,285 lbs of thawed turkey scap meat was 9.21 g [0.16 g (aqueous) + 5.51 g (gas) + 3.54 g (gas)].

The order of addition of ozone in this test was as follows:

The 1,285 lbs of turkey scap meat was added to the tumbler, followed with 257 lbs of water containing 0.16 g of ozone in total (procedure #1, above). The mixture was tumbled for approximately 66 minutes. During this time, ozone gas was added to the tumbler, by procedures #2 and #3, described above. Thus, the initial exposure of turkey scap meat to dissolved ozone is estimated to be approximately 2.74 x 10^{-7} g of ozone per g of turkey scap meat:

1,285 lbs x 454 g/lb = 583,390 g

0.16 g ozone) 5.834 x 10^5 g = 2.74 x 10^{-7} g O_3/g turkey scrap meat.

By addition of ozone in the gas phase following addition of the ozone-containing water, an additional 9.05 g of ozone was added to the meat. Thus, on average, the exposure of the turkey scap meat to this additional gaseous ozone was estimated to be:

9 g ozone) 5.834 x 10^5 g of turkey meat = 1.55 x 10^{-5} g ozone/g of turkey scap meat.

It can be seen that the amount of ozone to which the meat was exposed from the dissolved ozone was less than 100th the total amount of ozone to which the meat was exposed in the gas phase, on a per gram basis.

The tumbler was emptied and the meat was sampled and then boxed in two metal containers which were sealed and frozen. At the completion of the tumbling, it was noted that all of the ozonated water had been absorbed completely by the meat. There was a slight ozone odor in the tumbler when the door was opened, and the meat also smelled slightly of ozone as it was being packed. Samples thawed at a later date for analysis showed no odor of ozone, and no organoleptic changes in the meat quality could be detected by a taste panel.

It was also noted that the ozone-treated meat was lighter in color than turkey scap meat treated without the use of ozone. Also, workers noted that the customary slimy feel typical on tumbled poultry, especially on turkey meat with high bacterial contamination, was absent on the ozone-treated meat.

Bacteriological samples were taken as follows just after the test period:

1. Sample A was taken of turkey meat before the ozone test.

2. Sample B was taken of turkey meat at the entrance of the tumbler immediately after the test.

Both samples were held at 40EF or below at all times. Samples were frozen immediately by storing in bags cooled with dry ice until they could be transferred to a freezer. Two days later, the samples were allowed to thaw in a refrigerator, a portion of each sample was removed, weighed, and washed with 250 mL of sterile dilution/rinse water in 50 mL aliquots. After appropriate dilutions, the wash waters from each meat sample were examined for the presence of coliform bacteria, fecal coliform bacteria, standard plate count organisms, *Staphylococcus* bacteria and *Salmonella* bacteria. The results are shown in Table 2.1.05.

These results indicate that there were no fecal coliform organisms or *Salmonella* bacteria present either in the control or the ozone-treated samples. However, a distinct reduction in the quantity of coliform bacteria and aerobic and facultative anaerobic heterotrophic bacteria were observed in the sample taken after ozone treatment, compared to the control sample.

Finally, the number of *Staphylococcus* bacteria increased after treatment; this increase may be a consequence of the decrease in the number of competing bacteria.

Table 2.1.06 shows data obtained for total plate count, *E. coli* bacteria, and total coliforms obtained from various samples of the turkey meat exposed to ozone in the tumbler.

Table 2.1.05. Bacterial Analyses of Turkey Scap Meat - Control Samples vs Ozonated Samples (Caracciolo, 1990)

Bacteria Types	**Sample A (Control), counts/g**	**Sample B (Ozonated) counts/g**
Fecal Coliforms	Negative	Negative
Salmonella	Negative	Negative
Staphylococcus	9	23
Coliforms	19,753	13,594
Plate Counts	145,411	45,121

Based on the results of this test, ozone treatment of meat is indicated to decrease the presence of various species of bacteria. Additionally, these beneficial results were obtained by adding a total of 9.21 g of ozone to 1,285 lbs of turkey scap meat.

Test #2 - Whole Chickens

Another test was conducted utilizing several brands of commercially available chickens. Eight chickens were purchased for the study. A ninth chicken had been exposed 45 minutes to ozone about a week earlier (1.2 mg/L of ozone in water for 30 minutes), then frozen after treatment, and was added to this study.

All chickens were tested for *Salmonella* organisms; only one tested positive. Ozone testing began at 1004 hours, at which time four chickens were immersed in water initially containing 0.7 mg/L of dissolved ozone. Total immersion time was 45 minutes, with ozone being added

continually. During the 45-min testing time, the average ozone concentration (monitored every five minutes by DPD analysis; averaged over 45 minutes) was found to be 0.35 mg/L (1.12% by weight).

During this 45-min test, the water was stirred every 5 minutes. At the end of the 45-minute period, the four chickens were removed. At 1113 hours, two additional chickens were placed in the bath now containing 0.85 mg/L dissolved ozone at the start of the test. During the next 30 minutes [during which time fresh ozone was added continuously]. The average dissolved ozone concentration in the water was 0.35 mg/L over the 30 minute test period, during which the bath was stirred every five minutes. At the end of the 30 minutes, the two chickens were removed from the bath.

At 1157 hours, three additional chickens (including the one which had tested positive for *Salmonella*) were immersed in the water bath now containing 0.9 mg/L of dissolved ozone. Over the next 45 minutes, ozone was added continuously to the water bath, and the average dissolved ozone concentration was estimated to be 0.3 mg/L. As before, the bath was stirred every five minutes.

Table 2.1.06. Bacterial Counts on Ozonized Turkey Scap Meats (Caracciolo, 1990)

Point of Sampling	**Total Plate Counts**	***E. coli* Counts**	**Coliform Counts**
Raw scap meat (before ozone treatment)	3,400,000	< 10	TNC (too numerous to count)
Raw scap meat (after ozone treatment)	500,000	< 10	360,000 (estd.)
SWABS			
1. One ft into tumbler (one ft area)	70	< 10	< 10
2. First square from the nose of the tumbler (3 inch area)	10	< 10	< 10
3. Second square from the nose of the tumbler (3 inch area)	10	< 10	< 10
4. (359) Meat sample (one inch area) before tumble)	1800 (mold)	< 10	470 (spreader)
5. (458) Meat sample (one inch area) (before tumbling)	TNC	< 10	TNC

Point of Sampling	Total Plate Counts	*E. coli* Counts	Coliform Counts
6. First square from nose of tumbler (after tumble) (3 inch area)	TNC	< 10	TNC
7. Second square from nose of tumbler (after tumble)	TNC	< 10	TNC

The last three chickens were resubmitted for *Salmonella* testing; each tested negative. Specific data obtained for the last three chickens are given in Table 2.1.07.

Table 2.1.07. *Salmonella* Count on Contaminated Chicken - Before and After Ozone Treatment (Caracciolo, 1990)

Sample	*Salmonella* Count	Proteus Count
Before Ozonation	40 MPN/g	930 MPN/g
After Ozonation	< 3 MPN/g	9 MPN/g

The *Salmonella* results on the two chickens which had tested negative for *Salmonella* initially indicates that no cross-contamination occurred in the ozonation bath.

The data presented above clearly show the efficacy of dissolved ozone and gaseous ozone to reduce counts of microorganisms on the surface of whole chickens. Additionally, it is clear that poultry testing positive for *Salmonella* bacteria can be rendered negative by suitable immersion in ozone-containing water over 30- to 45-minute periods of time, during which the average dissolved ozone concentrations are approximately 0.3 to 0.5 mg/L.

To summarize the conditions of exposure of whole chickens to ozone dissolved in water:

> Whole, eviscerated chickens weighing about three pounds each (about 1,400 g each) were held under water which had an initial dissolved ozone concentration of about 0.9 mg/L. During the time of exposure (30 to 45 minutes), gaseous ozone (1.12% by weight) was being added to the water continuously, in order to maintain the dissolved ozone concentration at approximately 0.3 mg/L.
>
> Thus, the average exposure of the chicken meat so treated was approximately 1.22 mg ozone/g of chicken meat, calculated as follows:
>
> 190 L (ca 50 gal) x 0.4 mg/L = 76 mg ozone
>
> 76 mg ozone per 2,800 g chicken (two 3-lb chickens) = 0.027 mg ozone/g of chicken per minute x 45 min = 1.22 mg ozone per g of chicken.

2.1.2.2 Sheldon and Ball (1984a) – Chiller Water Ozonation

An ozone contact system was designed to ozonate chiller water. Ozone gas entered the bottom of the column countercurrent to the chiller water flow. Contactor efficiency parameters for potable water were determined at 8EC and included: percent ozone utilization (68.3%); total ozone utilization (4.12 mg/mL); applied ozone dosage (6.03 mg ozone/L liquid); aqueous ozone residual concentration (3.85 mg/mL); arid ozone consumption (0.27 mg/mL). These efficiency parameters indicate that the sparge tower design used in these-studies was efficient in solubilizing ozone.

Broiler carcasses were chilled in water at 7EC for 45 min with or without ozone (4.0-4.5 ppm ozone residual concentration). Chilling reduced the levels of aerobic and psychrotrophic plate counts, total coliforms, fecal coliforms, *E. coli* and *Salmonella* on all carcasses; however, counts on carcasses chilled in ozonated water were reduced more (0.20-1.06 log cycles) than non-ozonated control carcasses (0.14-0.54 log cycles). Ozone is more effective in killing bacteria washed from broiler carcasses than in killing bacteria remaining on the carcasses. Furthermore, ozone had no significant effect on the color (CTIE Lab L, a, b values), odor, taste, or lipid oxidation (TBA values) of treated broiler carcasses.

During an 11 day shelf life study at 4.4EC, aerobic plate counts were not significantly different between water and ozone chilled carcasses, although ozone chilled carcasses had consistently lower counts. About a 60% difference in psychrotrophic plate counts was detected between water and ozone chilled carcasses on each sampling day except day 4 where a 95% difference in treatment counts was found. These significant reductions in spoilage microorganisms following ozonation contributed to an extension in shelf life of at least 1.5 to 2.0 days.

A seven log cycle reduction in aerobic plate counts was observed in spent prechiller water ozonated for one hour. Fecal coliforms, *E. coli* and *Salmonella* were not detected in spent prechiller water after ozonation. The chemical oxygen demand (COD) was consistently reduced by 35% after one hour of ozonation while percent light transmission at 500 nm was increased about 30% to between 30 and 45% of the actual light transmission values of the poultry plants potable water. In other studies, light transmission increased above the USDA's 60% requirement for recycling chiller water.

Ozonation of poultry chiller water appears to be a suitable treatment process for reducing spoilage and pathogenic microorganisms on broiler carcasses and in chiller water without sacrificing broiler carcass quality. Moreover, ozone treatment under the right conditions fulfills the requirements of the USDA for recycling spent poultry chiller water.

Following the development of a satisfactory chiller system broiler carcasses were examined to determine the differences in microflora before and after chilling in either water or ozonated water. Water chilling alone produced slight, but consistent reductions in all counts (aerobic plate count [APC], 46%; psychrotrophic plate count [PPC], 28%; coliforms, 71%; fecal coliforms, 71%; *Salmonella*, 61%). Chilling in ozonated water also showed consistent reductions that were greater than chilling alone. With the exception of the PPC which had an average reduction of 37%, average reductions in all other microorganisms of greater than 75% were found in ozone-

treated carcasses. There was a 78% reduction in levels of APC, 91% in coliforms and fecal coliforms, and 99% in *Salmonella*.

Furthermore, no skin color differences on the breast, drumstick or back were detected between hot, water chilled or ozone chilled carcasses. Visually, an acceptable washing effect resulting in a loss of redness was noticed on the surface of both water and ozone chilled carcasses. No differences in lipid oxidation were found between the two treatments. With the exception of the drumstick where ozonated carcasses had slightly higher but acceptable TBA numbers than water chilled carcasses, all other ozonated carcass tissues (breast, thigh, back skin) had lower TBA numbers.

Sensory evaluations were conducted on the breast and thigh meats from each treatment by a 20 member triangle taste panel. Panelists -were unable to detect any flavor, off-flavor or texture differences between the two treatments. Thus, at the ozone concentrations and exposure times used in these studies, ozone effectively reduced the population of spoilage and pathogenic bacteria contaminating the carcasses, yet had no adverse effects on carcass quality characteristics.

A second objective was to determine shelf life differences at 4.4EC between water and ozone-chilled carcasses. Ozone-treated broiler carcasses consistently had lower APC and PPC on each Sampling day than water-chilled carcass. About a 60% treatment difference in PPC was observed on each sampling day except day 4, when ozonated carcasses had 95% fewer PPC. There was between a 1.5 to 2.0 day difference in the time required to reach a given PPC between water and ozone-chilled broilers. For example, 6.2 days were required for water-chilled carcasses as opposed to 7.3 days for ozone-chilled carcasses to reach a carcass PPC of log 8.0.

In the final set of experiments, spent poultry prechiller water from a commercial broiler processing facility was ozonated to determine the effects of ozone on several water quality characteristics. Microbial numbers were significantly reduced for each count following ozonation. About a seven log cycle reduction in APC was measured following ozonation. Coliform levels were reduced over three log cycles and were not detected in one third of the samples following ozone contact. Fecal coliforms, *E. coli*, and *Salmonella* were not detected after ozonation.

Percent light transmission at 500 nm (clarity) of treated chiller water was increased about 31% following ozonation to between 26 and 45% of the light transmission of the poultry plants potable water supply. In other studies conducted by the authors, light transmission at 500 nm exceeded the 60% minimum USDA requirement needed to recycle spent chiller water.

The COD of the spent poultry chiller water was consistently reduced by 33% despite the variation in initial CODs of the water. Oil and grease comprised about one third of the total solids of the spent prechiller water.

The findings of these final experiments demonstrate ozone to be quite effective in reducing levels of spoilage and pathogenic bacteria washed from the carcasses and effective in reducing levels of organic matter of chiller water to levels acceptable by the USDA for recycling.

Furthermore, the reduction of Salmonella levels in chiller water by ozone would reduce the threat of cross-contamination of *Salmonella* between carcasses during chilling.

Impact of the Findings for the Industry. Long and short range benefits:

Positive Findings: - This research has demonstrated that:

1. Chilling broiler carcasses in ozonated chiller water reduced the population of aerobes. psychrotrophs, coliforms, fecal coliforms, *E. coli*, and *Salmonella* contaminating the carcass surface.

2. Ozone had no adverse effects on broiler carcass color, lipid oxidation or on sensory quality (taste, odor, texture).

3. Ozone-chilled broilers had a projected extended shelf life of 1.5 to 2.0 days over conventionally chilled carcasses.

4. Ozone destroyed greater than 99% of the bacteria contaminating spent prechiller water.

5. Fecal coliforms, *E. coli*, and *Salmonella* could not be detected in ozonated spent prechiller water.

6. Ozone effectively reduced the COD and total solids levels in spent prechiller water while significantly improving the light transmission or clarity of the water.

Negative Findings: - This research also found that ozone was more effective in destroying bacteria washed from carcasses than in killing bacteria remaining on the carcasses.

Potential Usefulness: - Ozonation of poultry chiller water appears to be a suitable treatment process for reducing levels of spoilage and pathogenic microorganisms on broiler carcasses and in chiller water without sacrificing broiler carcass quality. Furthermore, the significant reduction of levels of *Salmonella* and other spoilage microorganisms in chiller water by ozone reduces the threat of cross-contamination between carcasses during chilling which ultimately reduces the number of reported food poisonings associated with poultry.

Potentially the greatest reward from this research is the knowledge about the effectiveness of ozone in treating spent process water. These studies have shown ozone to be effective in meeting the USDA's criteria for water recycling. Furthermore, these studies indicate that pretreatment of spent process water with ozone would aid in improving the efficiencies of other water treatment methods such as filtration and flotation. Thus, the final outcome would be water conservation, reduction in processing costs and improved quality of discharged water.

2.1.2.3 Sheldon et al. (1985a) – Spent Pre-chiller Water

The effects of ozone on the microbiological and quality characteristics of spent poultry prechiller water were investigated using two independent countercurrent ozone flow sparge towers.

Water Sample Collection

Approximately 95 L of spent prechiller (Gold Kist, Inc., Durham, NC) water were sampled from a fat flotation vat lying immediately downstream from the prechiller exit for use in the pilot plant sized contactor studies whereas 19 L were sampled directly from the overflow exit of the prechiller for use in the bench top size contactor. The water was filtered through five layers of cheesecloth to remove large particulates prior to treatment. Water samples used in determining (COD), % light transmission, total Kjeldahl nitrogen and microbial contamination loads were collected from the contactor exit ports in sterile 500 mL Erlenmeyer flasks.

Experimental Design

Experiment 1: Using the pilot plant size ozone contactor, approximately 94.6 L of spent prechiller water was ozonated for 60 min. Total aerobes, coliforms, *E. coli*, and *Salmonella* were enumerated prior to and after 60 min of ozonation. Chemical oxygen demand and % light transmission (%T) at 500 nm were determined after 0, 30, and 60 min of ozonation. Ozone was generated at a rate of approximately 292 ppm per min. Three experimental replicates were performed.

Experiment 2: Using the lab bench size ozone contactor, 9.5 L of spent prechiller water was ozonated for 50 min. Chemical oxygen demand, %T at 500 nm, total Kjeldahl nitrogen, total solids, and total aerobes were determined after 0, 10, 20, 30, and 50 min of ozonation. Ozone was generated at a rate of approximately 585 ppm per min. Three experimental replicates were performed.

Bacteriological Methods: Chiller water samples were serially diluted in 0.1% peptone water. Total aerobes (32EC, 48 hr) were enumerated using Plate Count Agar (Difco). Total coliforms, fecal coliforms and *E. coli* were enumerated by a 5-tube Most Probable Number (MPN) technique (1 mL sample size) using lauryl sulfate tryptose, brilliant green bile lactose and EC broths (BBL). Gas positive EC tubes were streaked on Levine's eosin methylene blue agar (BBL). Typical dark colonies with or without a metallic sheen were streaked on PCA slants before confirming using the IMViC classification.

Isolation and enumeration of *Salmonella spp.* was based on a 3-tube MPN using a 100, 10, 1 and 0.1 mL water sample size. To each dilution, an equal volume of double strength lactose broth with 1.2% Tergitol 7 was added. After 24 hr incubation at 37EC, 1 mL from each MPN tube was added to 10 mL selenite/cystine broth (BBL) (37E, 24 hr). A loopful from each selenite/cystine broth tube was streaked on xylose/lysine deoxycholate (XLD) agar (BBL) plates. Typical black colonies were streaked and stabbed onto triple sugar iron agar and lysine iron agar (Difco) slants. Positive cultures were identified using Roche Enterotube II. Counts for water samples are reported per 100 mL.

RESULTS & DISCUSSION

In experiment 1, the effects of ozonating spent prechiller water from a commercial broiler processing facility were investigated to determine the potential of meeting the USDA's criteria for water recycling. Table 2.1.08 summarizes the bactericidal effects of ozone on several groups

of microorganisms. Microbial numbers were reduced significantly in each bacterial category following ozonation. About a seven log cycle reduction in the total aerobic population was detected following ozonation. Coliform levels were reduced over three log cycles and were not detected in 1 out of 3 replicates after ozonation. Fecal coliforms, *E. coli* and *Salmonella* were not detectable following ozonation. These findings clearly demonstrate that ozone significantly reduces the population of chiller water microbial contaminants to an acceptable level that meets the USDA requirements for recycling.

Table 2.1.08. Log_{10} bacterial counts per mL of spent poultry prechiller water before and after ozonation (arithmetic mean of three replications) (Sheldon et al., 1985a)

	Before	**After** [d]	**Difference**
APC [a]	7.81	1.32	6.49
Coliforms [b]	3.45	-0.48	3.45
Fecal Coliforms [b]	3.45	ND [e]	3.45
E. coli [b]	3.45	ND	3.45
Salmonella [c]	-1.90	ND	-1.91

a Aerobic Plate Count per mL of chill water
b Most Probable Number, MPN per mL of chill water
c Most Probable Number, MPN per 100 mL of chill water
d 60 minutes of ozonation
e ND = Not detectable

No detectable aerobic microorganisms were found after 10 min of ozonation. This reduction represents a 4-log decrease in contaminating bacteria and exceeds the USDA's recycling requirements of a 60% reduction in levels of total microorganisms. The significant difference in the initial concentration of aerobes detected between experiments 1 and 2 reflect the installation of a new commercial prechiller at the processing plant prior to initiating experiment 2. Furthermore, in experiment 1, water was sampled from a fat flotation vat immediately downstream of the prechiller exit, whereas in experiment 2 samples were collected at the prechiller exit.

The results of this second study clearly indicate that ozone can be used effectively to improve the quality of spent poultry chiller water. Under the conditions of this study, treated chiller water would qualify for recycling under the USDA's guidelines. The water quality differences noted between experiments 1 and 2 are primarily due to differences in the ratio of ozone to water treated. The ratios were 3.1 and 61.5 ppm ozone per liter of water per min in experiments 1 and 2, respectively.

CONCLUSIONS: The results of this study support the findings of other researchers in that ozone can be effectively used to treat and disinfect wastewater from poultry processing plants. Under the conditions of this study, the quality of ozonated chiller water was improved beyond the USDA's water recycling requirements. Thus, the potential exists through ozonation for reducing the quantity of fresh water needed to chill poultry carcasses which should result in a cost savings for the processor.

2.1.2.4 Sheldon (1986) – Poultry Wastewater Treatment

The primary objective of this study was to investigate the effects of ozonation alone and in combination with different physical wastewater treatments for reconditioning spent poultry processing waters. The project focused primarily on broiler overflow prechiller water since it is the only process water that the USDA will allow to be recycled. However, a secondary objective was to explore the reconditioning opportunities of several other poultry processing waters such as whole bird rinse and neck chiller waters. After identifying suitable wastewater treatments to recondition these waters, hot broiler carcasses were chilled in either fresh potable water or reconditioned prechiller water meeting the USDA recycling requirements and their quality subsequently evaluated.

Several wastewater treatments were tested including direct ozonation, a combination of ozonation with either slow sand filtration, dissolved air flotation (DAF), rapid sand filtration, or diatomaceous earth (DE) filtration. The quality of overflow prechiller water was significantly improved with all treatments examined, surpassing the USDA recycling requirements in nearly all trials. Not only did the treatments satisfy the recycling requirements but removed significant portions of BOD, solids and fat, oil and grease (FOG). Thus the implementation of these treatments would reduce the effluent pollutant loads resulting in a reduction of wastewater surcharges.

Of all the treatments tested, a combination of prescreening, DE filtration and postozonation yielded the highest quality water. With this treatment significant reductions in chemical oxygen demand (COD), total solids (TS), FOG, total aerobic microorganisms, coliforms and *Salmonella* of 87%, 65%, 95%, 99.9%, 99.8%, and 99.9%, respectively, were detected. This water would qualify to be recycled back to the chiller at a rate of 1.1 gallons of reconditioned water for every 1 gallon of fresh water. Carcasses chilled in recycled prechiller water compared favorably to those chilled in fresh water. No significant differences in the carcass microflora including total counts, coliforms or *Salmonella* or in shelf life, taste or skin color were detected between carcasses from the two chilling methods.

The treatment reported on above was also tested on two additional sources of poultry processing water; whole bird rinse and neck chiller water. The quality of both waters was significantly improved by the wastewater treatment. Although the USDA has not approved the recycling of these waters, this study clearly demonstrates that based on the criteria used by the USDA for recycling chiller water, both whole bird rinse and neck chiller waters would qualify for recycling.

Reconditioned prechiller water meeting at least the minimum recycling requirements was used to chill hot broiler carcasses. Only water treated by the prescreening/DE filtration/postozonation treatment was to chill carcasses. Comparisons of carcass quality between these carcasses and carcasses chilled in fresh water were made. No significant treatment effects were detected between the two sets of carcasses. Analyses included evaluating the total bacterial population including coliforms, *E. coli*, and *Salmonella* as well as shelf life of carcasses held at 4.4EC. No differences in aerobic or psychrotrophic plate counts were found during the 13 day shelf life study. Furthermore, taste panelists were unable to detect any flavor differences between broiled breast fillets from each treatment. Lastly, no significant differences in skin color across the breast, leg or back were found between carcasses when examined with a color reflectance

spectrophotometer. Thus, these studies demonstrated that chilling broiler carcasses in reconditioned prechiller water did not adversely affect carcass quality.

2.1.2.5 Sheldon and Brown (1986) – Spent Chiller Water

Ozone Contact System – An ozone contact system was designed using a 3.35-m polyvinyl chloride (PVC) pipe (15.24 cm i.d. as a sparge tower. The water level was maintained at a 3.05-m level using a clear sight tube attached to the side of the column as a height indicator. Water was pumped at a 11. 35 L/min flow rate from an open 18.9-L polypropylene holding reservoir to the top of the column using a centrifugal pump with a sanitary relief value for throttling the flow rate. Water exited from the bottom of the column after a 5-min residence time and flowed to an open polypropylene product reservoir (either 18.9 or 113.6 L depending on the objective of the experiment). Overflow exiting the product reservoir was carried to the holding reservoir and recirculated back to the contactor. In an alternate arrangement, water bypassed the holding reservoir and was pumped directly from the product chamber to the contactor. To maximize the ozone transfer efficiency into water, non-solubilized gases exiting the top of the column were recycled back to either the holding or product reservoir. Ozone entered the bottom of the column through a stainless steel diffuser (2 micron pore size) countercurrent to the water flow.

Broiler Sample Collection – Approximately 2 hr after plant startup, hot broiler carcasses were collected from a commercial poultry processing plant just as they were to enter the prechiller. The carcasses were sealed in plastic bags and then packed in ice. Treatment began within 30 min of sampling. Carcasses were chilled in ice water for 45 min with or without ozone to an internal breast temperature of 7EC.

Water Sample Collection – Approximately 94.6-L of spent prechiller water were sampled and transported to N.C. State University. The water was filtered through five layers of cheesecloth to remove large particulates prior to treatment. Water samples used in determining bacteriological analysis were collected from the product chamber in sterile 500 mL Erlenmeyer flasks.

Experimental Design

Experiment 1. Two hot broiler carcasses were sampled using a whole bird rinse technique to determine total aerobes, psychrotrophs, total and fecal coliforms, *E. coli*, and *Salmonella*. CIE Lab color reflectance values were determined on the skin of the two hot carcasses following the whole bird rinse procedure. Chilled tap water (municipal water source, chlorinated) was preozonated for 25 min to obtain a 3.0-4.5 mg/L aqueous ozone concentration. Four hot broiler carcasses (two per treatment) were chilled in either ozonated tap water or tap water for 45 min using the 18.9-L product reservoir. Approximately 45.4 kg of crushed ice was added over this time period to maintain a 7EC (45EF) water temperature. Following chilling, the birds were hung on shackles and allowed to drain 15 min in a 4-4EC cooler. Microbes were enumerated and CIE Lab color values, TBA numbers and sensory evaluations determined on the carcasses. Three experimental replicates were performed. The data were analyzed by analysis of variance (AOV) for a split plot design and the Waller Duncan t test was used to determine significant mean differences.

Experiment 2. This experiment was designed to compare the shelf life of broiler carcasses chilled in ozonated water versus non-ozonated water. Water was preozonated for 25 min to obtain a 3.0-4.5 mg/L aqueous ozone concentration. Ten hot broiler carcasses were chilled in either ozonated water or water (control) for 45 min using the 113.6 L product reservoir. Approximately 68.0 kg of crushed ice was added to maintain a 7EC water temperature. After chilling, the carcasses were hung on shackles and allowed to drain 15 min in a 4.4EC cooler and individually packaged in oxygen permeable plastic bags. The birds were stored at 4.4EC and two carcasses sampled at 0 4, 7, 9, and 11 days to determine the total aerobes and psychrotrophs. Three experimental replicates were performed. The data were analyzed using AOV.

Experiment 3. Using the modified ozone contact system where water bypassed the holding reservoir, approximately 94.6-L of spent prechiller water was ozonated for 60 min. Total aerobes, coliforms, *E. coli*, and *Salmonella* were enumerated prior to and after 60 min of ozonation. Chemical oxygen demand and %T at 500 nm were determined after 0, 30 and 60 min of ozonation. Three experimental replicates were performed.

Bacteriological Methods: Individual broiler carcasses were rinsed in plastic bags with 100 mL sterile distilled water using a whole bird rinse technique. The rinses for two carcasses were combined (approximately 150 mL) and then serially diluted in 0.1 % peptone water. Chiller water samples were serially diluted in 0.1% peptone water. Total aerobes (32EC, 48 hr) and psychrotrophs (7EC, 10 days) were enumerated using Plate Count Agar (Difco). Total coliforms, fecal coliforms and *E. coli* were enumerated by a 5-tube Most Probable Number (MPN) technique (1 mL sample size) using lauryl sulfate tryptose, brilliant green bile lactose and EC broths (BBL). Gas positive EC tubes were streaked on Levine's eosin methylene blue agar. Typical dark colonies with or without a metallic sheen were streaked on PCA slants before confirming using the IMViC classification. All counts were reported on a per mL of rinse water or chiller water basis.

Isolation and enumeration of *Salmonella spp.* were based on a 3-tube MPN using a 100 mL rinse water sample size for the carcasses and a 100, 10, 1, and 0.1 mL water sample size. To each dilution, an equal volume of double strength lactose broth with 1.2% Tergitol-7 was added. After 24 hr incubation at 37EC, 1 mL from each MPN tube was added to 10 mL selenite cystine broth (BBL) (37EC, 24 hr). A loopful from each selenite cystine broth tube was streaked on xylose lysine deoxycholate (XLD) agar (BBL) plates. Typical black colonies were streaked and stabbed onto triple sugar iron agar and lysine iron agar (Difco) slants. Positive cultures were identified using Roche Enterotube II. *Salmonella* counts were reported per 100 mL of rinse water or chiller water.

RESULTS & DISCUSSION

In Experiment 1, broiler carcasses were examined to determine the differences in microflora before and after ozonation. No significant replicate differences were noted among hot carcasses for both treatments prior to chilling. Therefore, the hot carcass counts were pooled and averaged for comparison with the water and ozone treatment mean carcass counts (Table 2.1.09).

Table 2.1.09. Mean $\log_{10}$ (range) bacterial count per carcass of hot, water and ozonated carcasses (experiment 1)[a] (Sheldon and Brown, 1986a)

Microorganisms	Hot water	Water	Ozonated Water
APC [b]	6.22 (4.56, 6.72)	5.95 (4.89, 6.38)	5.56 (4.56, 6.00)
PPC [c]	5.23 (3.41, 5.69)	5.09 (3.74, 5.54)	5.03 (3.41, 5.48)
Coliforms [d]	4.27 (2.90, 4.38)	3.73 (3.23, 3.96)	3.21 (2.98, 3.38)
Fecal Coliforms [d]	4.27 (2.90, 4.38)	3.73 (3.23, 3.96)	3.21 (2.98, 3.38)
Salmonella [e]	1.36 (0.48, 1.66)	1.08 (0.48, 1.38)	0.64 (-0.04, 0.97)

[a] Arithmetic mean of three experimental replicates (two birds per replicate)
[b] Aerobic Plate Count, colony forming units per mL of rinse water
[c] Psychrotrophic Plate Count, colony forming units per mL of rinse water
[d] Most Probable Number, MPN/mL of rinse water
[e] Most Probable Number, MPN/100 mL of rinse water

There were significant replicate differences within the water and ozone treatments for both the aerobic (APC) and psychrotrophic (PPC) plate counts. Compared to hot carcass counts, water chilling alone produced slight, but consistent reductions in all counts (APC, 46%; PPC, 28%; coliforms, 71%; fecal coliforms, 7 1 %; *Salmonella*, 48%). Chilling with ozonated water also showed consistent reductions that were slightly greater than water chilling alone. With the exception of the PPC which had an average reduction of 37%, average reductions of greater than 75% were observed between hot and ozone-treated carcasses for each count. There was a 78% reduction in levels of APC, 91 % in coliforms and fecal coliforms and 81 % in *Salmonella*.

Statistically, treatment differences for each mean log bacterial .count were significant at $p<0.07$. It was observed that the total coliform, fecal coliform and *E. coli* counts were always identical within a replicate. The total and fecal coliform counts would be expected to be similar because fecal contamination is common during poultry processing operations. It has been shown (Barnes and Impey, 1968) that bacteria on poultry carcasses primarily are located on exposed muscle surfaces and within feather follicles on the skin. Those microorganisms located within the feather follicles generally are protected from the bactericidal action of disinfectants as shown by relatively small reductions in carcass microbial numbers resulting from washing and ozonation. The aqueous ozone residual concentration was 4.0-4.5 mg/L as the carcasses were added to the water. After 25 min of chilling, 2.1 mg/l ozone still could be measured. These findings suggested the need for better mixing or contact of the carcasses with ozone to achieve greater reductions in levels of surface bacteria.

In experiment 2, broiler carcasses were examined to determine differences in shelf life at 4.4EC between water-chilled and ozone-chilled carcasses. With the exception of the initial PPC, ozone-

treated broiler carcasses had consistently lower APC and PPC on each sampling day than water-chilled carcasses (Table 2.1.10). The APC between treatments were not significantly different over the 11-day sampling period, although there was a significant treatment effect for PPC ($p<0.05$). About a 60% difference between treatments was observed in PPC on each sampling day except on day 4, when a 95% difference in counts was detected. However, by the end of the sampling period (11 days) the mean ozone PPC was nearly identical to the mean water-chilled carcass PPC. As expected, APC increased at a lower rate than PPC during refrigeration.

Table 2.1.10. Effect of chill water and ozonated chill water on the shelf life of broilers stored at 4.4EC (experiment 2)[a] (Sheldon & Ball, 1986a)

Day	Treatment			
	Water		Ozone	
	APC [b]	PPC [c]	APC	PPC
0	4.93 (4.78-5.11)	3.73 (3.66-3.77)	4.54 (4.00-4.83)	3.92 (3.51-4.20)
4	4.96 (4.64-5.26)	5.24 (4.64-5.54)	4.58 (4.41-4.72)	3.96 (3.87-4.04)
7	5.03 (4.64-5.28)	6.47 (6.18-6.54)	5.01 (4.95-5.08)	6.17 (5.32-6.57)
9	5.14 (4.89-5.28)	7.56 (7.30-7.82)	5.11 (4.89-5.28)	7.09 (6.11-7.38)
11	6.08 (5.24-6.30)	9.00 (8.23-9.28)	6.00 (5.48-6.34)	8.58 (7.56-9.00)

[a] Arithmetic mean of three experimental replicates (two birds per sampling time per replicate)
[b] Aerobic Plate Count, $\log_{10}$ (range) colony forming units per mL of rinse water
[c] Psychrotrophic Plate Count, $\log_{10}$ (range) colony forming units per mL of rinse water

From an appearance and sensory standpoint, neither the water-chilled nor ozone-chilled broiler-carcasses were spoiled after 11 days. Appearance (slime formation) and odor were evaluated subjectively by a group of four experienced panelists working in the poultry research section of the N.C. State University Food Science Dept. Using a conversion factor of 1900 cm^2 for a 2.5 pound (1135 g) broiler, the 11-day mean $\log_{10}$ aerobic and psychrotrophic plate counts on a per cm^2 basis would be 4.80 and 7.72 for the water-chilled carcasses and 4.72 and 7.30 for the ozonated carcasses, respectively. Broilers used in this study ranged in weight from 992 g to 1275 g. Ayres et al. (1950) detected the presence of off-odors and slime formation on broiler carcasses stored at 4.4EC when aerobic plate counts had reached a $\log_{10}$ count of over 8.0 per cm^2. However, they point out that shelf life is very dependent on the initial level of microorganisms present with lower initial levels yielding a longer shelf life. Other disinfection studies (Thiessen et al., 1984) have shown that a small decrease in the initial carcass counts which resulted from chlorine dioxide treatment (about 0.5 log cycle) lengthened shelf life by around 2 days when compared to control carcasses. In the present study, there was a 0.3 to 1.28 log difference in the PPC between water- and ozone-chilled broilers for days 4, 7, and 9. However, the exact point of spoilage was not identified for either treatment. The aqueous ozone residual concentrations before the broiler carcasses were added to the water was 3.7-4.2 mg/L and 2.2-2.6 mg/L after 25 min of chilling.

In experiment 3, spent prechiller water from a commercial broiler processing facility was treated with ozone to determine the effects of ozone on the potential of recycling chiller water in poultry processing plants. The microbial counts for the ozonated spent prechiller water are

shown in Table 2.1.11. Microbial numbers were significantly reduced for each count following ozonation. About a seven log cycle reduction following ozonation was observed for APC. Coliform levels were reduced over three log cycles and were not detected in 1 out of 3 replicates after ozonation. Fecal coliforms, *E. coli* and *Salmonella* were not detected after ozonation using MPN procedures.

These findings are highly significant considering that commercial chiller water bacterial numbers increase throughout the day. Findings by Sheldon and Brown (1986a) showed that ozone would be effective in reducing chiller water bacterial numbers and therefore carcasses introduced throughout the day might undergo loss cross-contamination. Furthermore, cross-contamination of *Salmonella* between carcasses in chillers may be minimized. Since *Salmonella* numbers were extremely low prior to ozonation, further work is needed employing larger sample sizes to determine the bactericidal effects of ozone in chiller water on this group of microorganisms. However, studies recently completed by these authors have shown a 7-log cycle reduction of *Salmonella typhimurium* in physiological saline solution following 30 sec of ozonation.

Sheldon and Brown (1986a) concluded that the USDA requirements for microbial reductions for chiller water reuse were achieved consistently in this study using ozone. About one-half the required increase in percent light transmission was achieved using ozone. The remainder of the required increase in light transmission could be achieved by using a more favorable ozone to water ratio or by employing other treatment methods such as filtration with diatomaceous earth, activated carbon, flotation cells or centrifugal waste concentration. The findings of this study demonstrated ozone to to be a suitable treatment process for reducing spoilage and levels of pathogenic microorganisms on broiler carcasses and in chiller water without sacrificing broiler carcass quality. Moreover, ozone-treated chiller water partially fulfills the requirements of the USDA for recycling, and therefore merits further investigations.

Table 2.1.11. Log_{10} bacterial counts per mL of spent poultry prechiller water before and after ozonation (experiment 3) [a] (Sheldon & Brown, 1986a)

Microorganisms	Replicate			
	1	**2**	**3**	**Mean**
APC [b]				
before	8.20	7.17	7.26	7.81
after	0.65 est [e]	1.38 est	1.53	1.32
Coliforms [c]				
before	3.54	3.11	3.54	3.45
after	ND [f]	-0.40	-0.40	---
Fecal Coliforms [c]				
before	3.54	3.11	3.54	3.45
after	ND	ND	ND	---
E. coli [c]				
before	3.54	3.11	3.54	3.45
after	ND	ND	ND	---
Salmonella [d]				
before	2.18	2.18	1.88	2.10
after	ND	ND	ND	---

[a] Arithmetic mean of three experimental replicates
[b] Aerobic Plate Count per mL of chill water
[c] Most Probable Number, MPN/mL of chill water
[d] Most Probable Number, MPN/100 mL of chill water
[e]...EST = estimated
[f]...ND = not detectable

2.1.2.6 Sheldon and Chang (1987a) – Chiller and Neck Chiller Water

One objective of this study was to test the efficacy of three wastewater treatments to recondition spent overflow chiller water from broiler processing plants. Additionally, whole bird rinse water and neck chiller water were reconditioned using the most effective treatment process identified. The manner of applying ozone and collection of water samples were as described in previous publications by Prof. Sheldon and his colleagues.

Experimental Design

Experiment 1. This experiment was conducted to determine the effects of ozonation of prechiller overflow water on selected water quality characteristics [pH, chemical oxygen demand (COD), percent light transmission at 500 nm (%T), absorbance at 280 nm (ABS), total solids (TS), fats/oil/grease (FOG), ammonia (NH_4^+), aerobic plate count (APC), coliforms (COLIF), *E. coli*]. Using a laboratory bench size ozone contactor, 9.5 liters of prechiller overflow water was

ozonated for 50 min with samples taken at 0, 10, 20, 30, and 50 min of ozonation. The average ozone generator output was 18.1 ppm. Four experimental replicates were completed.

Experiment 2. The effects of ozonation in combination with rapid sand filtration of prechiller overflow water were tested on selected water quality characteristics including COD, %T, ABS, TS, total volatile solids (TVS), total fixed solids (TFS), and APC. Using a water reconditioning scheme, 160 liters of spent prechiller water were initially prefiltered through 5 layers of cheesecloth, preozonated for 15 min in a pilot plant contactor [ozone generator (32.4 ppm O_3, 86 L/min water flow rate), recycled through a high rate sand filter (0.162 sq m filtration area, 63 kg #20 silica sand: 0.046-0.056 cm diameter) for either 5 or 15 min at a flow rate of 95 L/min, and ozonated for 10 min using the preozonation conditions. Samples were taken after cheesecloth filtration, preozonation, 5 and 15 minutes of sand filtration, and after the final ozonation step. The ozone contactor, filter, pumps and all pipes and connections were sanitized with 10% chlorox for 15-20 min then rinsed with potable water for 15-20 min prior to use. This experiment was replicated once.

Experiment 3. The objective of this experiment was to evaluate the effects of DE filtration of overflow prechiller water followed by postozonation on COD, %T, ABS, NH_4^+, TS, FOG, APC, COLIF, *E. coli*, and *Salmonella* counts (SALM). One hundred sixty liters of spent water were recycled for 5 min through a Hayward model EC-50 Perflex DE filter at a flow rate of 159 liters per min. The filter f lex-tubes were precoated with 0.09 kg DE (2.25 kg total Celatom diatomite) per 0.09 m^2 filter area (resulting in a flow rate of around 1.9 L/min/0.09 m^2 of filter area. Following filtration the water was ozonated for 15 min in the pilot plant size contactor. Water was sampled before and after cheesecloth filtration and after ozonation. All filtration and ozonation equipment was sanitized prior to each run as described above. This experiment was replicated three times.

Experiment 4. Using the DE filtration and postozonation scheme outlined under experiment 3, 160 liters of spent whole carcass rinse water obtained from a commercial poultry processing plant on three separate days were treated using the operational conditions described in experiment 3. Selected water quality characteristics including COD, %T, ABS, TS, TVS, TFS, APC, COLIF, *E. coli* and *Salmonella* counts were tested on waters sampled before and after cheesecloth filtration and after the ozonation step.

Experiment 5. This final experiment was a duplicate of experiment 4 with the exception that spent broiler neck chiller water from the commercial poultry plant was treated on three separate days using the same treatments and operating parameters as in experiments 3 and 4. DE filtration was extended to 10 min to facilitate the effective removal of the higher waste loads.

Bacteriological Methods Process water samples were serially diluted in 0.1% peptone water. Total aerobes (32EC, 48 hr) were enumerated using Plate Count Agar (Difco). Total coliforms and *E. coli* were enumerated by a 5-tube most probable number (MPN) technique (1 mL sample size) using lauryl sulfate tryptose, brilliant green bile lactose and EC broths (BBL). Gas positive EC tubes were streaked on Levine's eosin methylene blue agar (BBL). Typical dark colonies with or without a metallic sheen were streaked on PCA slants before confirming using the IMViC classification. Eleven counts were reported on a per mL of water basis.

Isolation and enumeration of *Salmonella spp.* was based on a 3-tube MPN technique using 100, 10, 1 and 0.1 mL water sample sizes. To each dilution, an equal volume of double strength lactose broth with 1.2% Tergitol 7 was added. After 24 hr incubation at 37EC, 1 mL from each MPN tube was transferred to 10 mL selenite/cystine broth (BBL) (37EC, 24 hr). A loopful from each selenite/cystine broth tube was streaked on xylose/lysine deoxycholate (XLD) agar (BBL) plates. Typical black colonies were streaked and stabbed onto triple sugar iron agar and lysine iron agar (Difco) slants. Positive cultures were identified using Roche Enterotube II test kits. *Salmonella* counts were reported per 100 mL of water.

RESULTS AND DISCUSSION

The quality of chiller overflow water was significantly improved following ozonation (Table 2.1.12). All requirements for recycling as established by the USDA were met within 10 min of ozonation. That is, percent light transmission at 500 nm was at least 60% of fresh potable water (65.2%) and there was at least a 60% reduction in total microorganisms including total plate counts, coliforms, *E. coli*, and *Salmonella*. Reductions in bacterial levels of 3.43 logs or 99.96% of the aerobic microflora were killed after 10 min of ozonation in addition to the complete elimination of coliforms and *E. coli*. In addition to these findings the quality of water was significantly improved in all water quality categories examined.

The pH of the water decreased from 6.88 to 5.6 after 50 min of ozonation indicating the severe oxidative destruction of lipids and their associated aldehydes as well as the oxidative and potentially hydrolytic destruction of proteins. The findings of these experiments demonstrate that ozone is effective in improving the quality of spent chiller water to a degree suitable for recycling.

The objective of the remaining experiments was to examine the efficacy of several wastewater treatment schemes combining ozonation with other physical wastewater treatments on reconditioning poultry process waters. The first study combined preozonation, rapid sand filtration and postozonation using a pilot plant system (Table 2.1.13). Similar to the findings of the previous experiment, preozonation significantly improved the water quality after 15 min, yet would not qualify for recycling due to the inadequate %T (55.2%). Recycling of water through the sand filter for 5 or 15 min improved the water quality to beyond the recycling requirements for %T yet did not achieve the desired reductions in microbial levels. In fact, passage of water through the sand reinfected the water to concentrations approaching the cheesecloth filtered control water. The 10 min postozonation step was required to sufficiently reduce the microbial loads to satisfactory levels (3.23 log reduction or 99.9% kill).

Table 2.1.12. Effect of ozonation of spent chiller water on several water quality parameters (Sheldon and Chang, 1987a)

Ozonation time (min) [a]	Water Quality Parameters [b]			
	pH	APC, CFU/mL	Colifs, CFU/mL	*E. coli*, CFU/mL
0	6.88	4.39	2.42	2.42
10	6.68	0.96	0	0
20	6.40	0.41	0	0
30	6.18	0	0	0
50	5.60	0	0	0

[a] Ozone generator output: 18.1 ppm
[b] APC = aerobic plate count, geometric means

Table 2.1.13. Effect of ozonation in combination with rapid sand filtration of spent prechiller water on water quality (1 replicate) (Sheldon and Chang, 1987a).

Treatments [a]	APC, CFU/mL [b]
Control	4.82
O_3 - 15 min	2.26
O_3 - 15 min/filtration - 5 min	4.81
O_3 - 15 min/filtration - 15 min	4.80
O_3 - 15 min/filtration - 15 min/O_3 - 10 min	1.59

[a] Control: post cheesecloth screening; O_3 : generator concentration, 32.25 ppm
[b] APC - geometric means

Although significant improvements in water quality were achieved with this last treatment, it was felt that economics would dictate using another approach in treating chiller water. The high concentration of organic matter in spent prechiller water reduces the effectiveness of ozone as a disinfectant which results in increased ozone demands and therefore higher operating costs. Thus, the remaining experiments employed ozonation as a final disinfection step preceded by a 5 min DE filtration step.

The objective of the next series of experiments was to treat spent prechiller water by first passing the water through cheesecloth followed by recycling through a DE filter for 5 min and a final 15 min postozonation step (Table 2.1.14). Significant reductions in COD, ABS, TS, FOG, APC, COLIF, *E. coli*, and *Salmonella* of 87%, 85%, 65%, 95%, 99.92, 99.82, 99.7% and 99.9%, respectively, were detected after the filtration and disinfection steps. The %T values averaged 97% following treatment and easily met the recycling criteria as did the reduction in microbial loads. A comparison of these findings to the previous study indicate that DE filtration is significantly more effective than sand filtration in removing contaminants from wastewater and requires a shorter processing time. Both studies demonstrate the necessity for a final disinfection step following filtration. The initial filtration through cheesecloth was effective in significantly reducing the COD (50%), TS (35%) and FOG (74%) in chiller water.

Table 2.1.14. Effect of ozonation in combination with Diatomaceous Earth (DE) filtration on reconditioning spent broiler prechiller water (Sheldon and Chang, 1987a)

Treatments [1]	Water Quality Parameters [2]			
	APC, CFU/mL	Coliforms, MPN/mL	*E. coli*, MPN/mL	*Salmonella*, MPN/mL
Control 1	3.88 [a]	---- [3]	----	----
Control 2	3.95 [a]	3.00 [a]	2.81 [a]	1.38 [a]
DE	4.52 [a]	----	----	----
DE/O_3	0.53 [b]	< 0.30 [b]	< 0.30 [b]	< -1.52 [b]

[1] Control 1: before treatment; Control 2: after cheesecloth screening; DE: 5 min filtration; DE/O_3: 5 min filtration, 15 min postozonation, 30.4 ppm generator output.

[2] Mean of 3 replicates: APC, Coliforms, *E. coli, Salmonella* = geometric means.

[3] No data.

[abc] Means followed by different letter superscripts are significantly different, $P < 0.01$.

The final set of experiments examined the efficacy of DE filtration (5 min) followed by ozonation (15 min) on reconditioning spent broiler neck chiller water (Table 2.1.15) and spent whole bird rinse water (Table 2.1.16). Neck chiller wastewater was considerably more contaminated than the whole bird chiller water as reflected in the 2.5-fold increase in neck chiller water COD than whole carcass chiller water COD. Even with the higher initial waste loads, the reconditioning treatment tested in this study was very effective in improving the quality of the water to meet the USDA chiller water recycling criteria. Significant reductions in levels of COD (79%). ABS (72%), TS (52%), TVS (59%), APC (99.5%), COLIF (99.52), E. coli (99.5%) and SALM (99.9%) were detected following the treatment. Light transmission increased to 80.1% which would qualify the water to be used at a recycling rate of 1.1 gallons of reconditioned water for every 1.0 gallon of fresh water.

Table 2.1.15. Effect of DE filtration in combination with ozonation on reconditioning spent broiler neck chiller water (3 replicates) (Sheldon and Chang, 1987a)

Treatments [1]	Water Quality Parameters [2]			
	APC, CFU/mL	Coliforms, MPN/mL	*E. coli*, MPN/mL	*Salmonella*, MPN/mL
Control 1	4.41 [a]	---- [3]	----	----
Control 2	4.37 [a]	2.57 [a]	2.57 [a]	0.38 [a]
DE filtration/ ozonation	1.0 [b]	< 0.3 [b]	< 0.3 [b]	-2.52 [b]

[1] Control 1: before cheesecloth screening; Control 2: post cheesecloth screening; DE filtration-10 min/ozonation - 15 min.

[2] Microbial counts expressed as geometric means.

[3] No data.

[ab] Means with different letter superscripts are significantly different ($P < 0.1$).

Table 2.1.16. Effect of DE filtration in combination with ozonation on reconditioning spent broiler whole bird rinse water (3 replicates) (Sheldon and Chang, 1987a)

Treatments [1]	Water Quality Parameters [2]			
	APC, CFU/mL	Coliforms, MPN/mL	*E. coli*, MPN/mL	*Salmonella*, MPN/mL
Control 1	4.76 [a]	---- [3]	----	----
Control 2	4.75 [a]	2.32 [a]	2.32 [a]	0.38 [a]
DE filtration/ ozonation	0 [b]	< 0.30 [b]	< 0.30 [b]	-2.52 [b]

[1] Control 1: before cheesecloth screening; Control 2: post cheesecloth screening; DE filtration- 5 min/ozonation - 15 min; 30.4 ppm generator output.

[2] Microbial counts expressed as geometric means.

[3] No data.

[abc] Means with different letter superscripts are significantly different ($P < 0.1$).

The final study was a duplicate of the previous experiment except that whole bird rinse water was subjected to the reconditioning treatment (Table 2.1.16) Of all the waters examined in these studies, the whole bird rinse water was of the best quality and contained the least amount of contaminants. The quality of this water was significantly improved by passage through the pilot wastewater treatment. Significant reductions in levels of COD (92%), ABS (88%), TS (59%), TVS (82%), APC (100%), COLIF (99%), *E. coli* (99%), and *Salmonella* (99.9%) were detected in the treated water. Furthermore, the clarity of the treated water resembled that of fresh water and had a %T of 99.6%. The treated water would thus qualify for recycling in the event that the USDA were to permit its reuse. The approximate volume of water used to rinse each broiler carcass is 0.79 gallons or about 6% of the total water usage. The most effective treatment evaluated consisted of an initial screening through cheesecloth followed by DE filtration and postozonation.

2.1.2.7 Chang and Sheldon (1989a) – Broiler Process Waters

Several wastewater treatments, including direct ozonation and a combination of ozonation with either slow sand filtration, dissolved air flotation, or diatomaceous earth (DE) filtration, were tested for their ability to recondition broiler process waters taken from a commercial poultry processing plant. The quality of broiler prechiller overflow water was significantly improved with all wastewater treatments examined, surpassing the USDA's recycling requirements in nearly all trials. A combination of screening, DE filtration, and ozonation yielded the highest quality water. With this treatment, significant reductions in chemical oxygen demand, total solids, and the total microbial load including coliform bacteria and *Salmonella* of 87, 65, and 99.9%, respectively, were detected in the reconditioned prechiller water. Percentage of light transmission at 500 nm (%T) of treated water increased to 97% of tap water and compared favorably with %T of potable water. This same water treatment was tested on final carcass rinse and neck chiller overflow waters. The quality of these treated waters was also significantly improved. It was concluded that poultry process waters could be effectively reconditioned for recycling by screening, DE filtration, and ozonation. Furthermore, wastewater organic loads

discharged to wastewater treatment facilities can be effectively reduced through these reconditioning practices. Experimental details are as described in previous studies by Prof. Sheldon and his colleagues.

Experimental Design

Experiment 1. This experiment was designed to determine the effects of ozonation of prechiller overflow water on selected water quality characteristics. Using a lab bench-size ozone contactor and ozone generator, 9.5 L of prechiller overflow water were ozonated for 50 min with samples taken at 0, 10, 20, 30, and 50 min of ozonation. The COD, percentage of light transmission (%T, at 500 nm), absorbance at 280 nm (ABS), pH, ammonium ion (NH_4^+), total solids (TS), total volatile solids (TVS), total fixed solids (TFS), FOG, total aerobic plate count (APC), coliform bacteria (COLIF), *E. coli*, and *salmonellae* (SALM) were determined at the designated sampling times. The average ozone concentration generated was 1.37%. Four experimental replicates were completed, using water sampled on separate days.

Experiment 2. The objective of this experiment was to evaluate the effectiveness of ozone alone or in combination with sand filtration on reconditioning prechiller overflow water. Six treatments were evaluated: a control or nontreated sample; an ozonated sample (10 min, 1.37% ozone, ozone contactor as described in Experiment 1); a filtered sample; an ozonated (10 min)/filtered sample; a filtered/ozonated (5 min, same ozonation conditions as above) sample; and an ozonated (10 min)/filtered/ozonated (5 min) sample. The COD, %T, ABS, NH_4^+, TS, TVS, TFS, and APC were determined. The *E. coli* and *Salmonella* were not enumerated, given the consistent reductions obtained in previous ozonation studies (Sheldon and Brown, 1986). This experiment was replicated three times using water sampled on separate days.

Experiment 3. This experiment was designed to compare the effects of ozonation in combination with DAF of broiler prechiller overflow water on selected water quality characteristics including COD, %T, ABS, NH_4^+, TS, TVS, and TFS. A lab bench-size DAF apparatus was charged with 1.5 L of nonscreened prechiller overflow water and either compressed air (control) or ozone purged through the column via a 2-Φm pore size stainless steel diffuser for 3 min at a flow rate of 0.0015 m^3/min. Ozone was generated using the operating parameters described above. The chamber was immediately pressurized to 414 kPa with air, agitated by inversion for 1 min, and allowed to stand for 3 min. The pressure was then released and the sample was rested for an additional 10 min, after which 1 L of water was taken from the bottom port. No flocculants were used in these studies.

Four treatments were evaluated: a control or nontreated water, ozonation (3 min ozonation, 1.39% ozone, 0.005 m^3/min gas flow); DAF using compressed air (0.005 m^3/min gas flow); and DAF using ozone (3 min ozonation, 1.39% ozone, 0.005 m^3/min gas flow). This experiment was replicated three times using water sampled on separate days.

Experiment 4. The objective of this experiment was to evaluate the effect of DE filtration of broiler-processing overflow waters followed by ozonation. Approximately 160 L of prechiller, final carcass rinse, and neck chiller overflow waters were initially screened through five layers of cheesecloth and then recirculated through a DE filter for either 5 min (prechiller and final carcass rinse waters) or 10 min (neck chiller water) at a flow rate of 159 L/min. Preliminary studies

indicated that a 5-min DE filtration time was necessary to achieve adequate water quality. Following filtration the water was ozonated for 15 min in the pilot plant-size ozone contactor (2.30% ozone, 86 L/min water flow rate). Water was sampled before and after cheesecloth screening and after ozonation. Filtration and ozonation equipment was presanitized with 10% chlorox for 15 to 20 min then rinsed with potable water for 15 to 20 min prior to each run.

The APC, COLIF, *E. coli*, and *Salmonella* were enumerated prior to and after treatment. The COD, %T at 500 nm, ABS at 280 run, TS, TVS, TFS, FOG, and NH_4^+ also were determined before and after treatment. This experiment was replicated three times using water sampled on separate days.

RESULTS AND DISCUSSION

Experiment 1. The quality of prechiller overflow water improved following ozonation (Table 2.1.17). After 10 min of ozonation a 3.4 $\log_{10}$ or 99.96% reduction in the aerobic plate count was achieved. The COLIF and *E. coli* levels were reduced by over 2 $\log_{10}$ in the treated water. Significant reductions of 48, 19, and 76%, respectively, were detected for COD, TS, and FOG after 10 min of ozonation.

Table 2.1.17. Effect of ozonation of overflow prechiller water on several water quality parameters[1] (Chang and Sheldon, 1989a)

Ozonation time (min) [2]	pH	APC [3] CFU/mL	Coliforms MPN/mL	*E. coli* MPN/mL
0	6.88 [A]	4.4 [A]	2.4 [A]	2.4 [A]
10	6.68 [AB]	1.0 [B]	< 0.3 [B]	< 0.3 [B]
20	6.40 [BC]	0.4 [B]	< 0.3 [B]	< 0.3 [B]
30	6.18 [C]	0 est [B]	< 0.3 [B]	< 0.3 [B]
50	5.60 [D]	0 est [B]	< 0.3 [B]	< 0.3 [B]

[A-D] Means within the same column with no common superscripts are significantly different ($P < 0.01$), n = 8.

[1] APC = aerobic plate count, colony forming units per mL ($\log_{10}$); MPN = most probable number ($\log_{10}$).

[2] Ozone generator output: 1.37% ozone.

[3] 0 est = no culturable microorganisms detected.

Experiment 2. The results of sand filtration and ozonation on prechiller overflow water are presented in Table 2.1.18. Ozonation for 10 min significantly improved water quality by reducing levels of COD by 47%, ABS by 51%, TS by 24%, and APC by 99.6%. Passage of non-ozonated water through the sand filters resulted in no significant reduction in the microbial loads, illustrating that sand filtration is not adequate for removing the microflora.

The quality of water was further improved by combining pre or postozonation with sand filtration (Table 2.1.17). The highest water quality in this treatment was obtained when ozonation preceded and followed the filtration stage, where significant reductions in levels of

COD (92.8%), ABS (93.4%), NH_4^+ (63.6%), TS (60.2%), and APC (99.97%) were obtained. A sufficient reduction in microbial loads, which satisfied the USDA recycling criteria, was achieved only when the filtration step was followed by ozonation. However, the sand bed was presanitized only at the beginning of each experiment. The increase in APC of the ozonation/filtration treatment in comparison with the ozonation treatment is attributed to the fact that the ozonation/filtration treatment followed the filtration treatment without sanitizing between treatments.

Experiment 3. This study combined ozonation with DAF to recondition overflow prechiller water. The DAF alone yielded improvements in water quality characteristics similar to those of ozonation with the exception of ABS.

Experiment 4. Preliminary studies (data not shown) were conducted to establish the optimal DE filtration and ozonation times. These findings demonstrated that each successive pass through the DE filter improved the %T of the filtered water, reaching 94.2% after 5 min or the equivalent of 5.03 passes. Continued recirculation of the water through the filter for 10 and 15 min resulted in only minor COD and TS reductions beyond those achieved at 5 min. After 15 min of ozonation the total aerobic microbial population was reduced to less than 1 cfu/mL of treated water. Although a 98% reduction in the aerobic plate count was achieved after 5 min of ozonation, it was felt that 15 min of ozonation would provide a safeguard for any anticipated daily fluctuations in water quality. These results confirmed that a combination of prescreening, DE filtration, and ozonation treatments would be suitable for reconditioning overflow prechiller water.

Table 2.1.18. Effect of ozonation (O) and sand filtration (F) on the quality of overflow prechiller water (Chang and Sheldon, 1989a)

Treatment [2]	**APC (CFU/mL)**
Control	4.5 [A]
O	2.0 [B]
F	5.3 [A]
O/F	4.0 [A]
F/O	1.9 [B]
O/F/O	1.0 [B]

[A-D] Means within the same column with no common superscripts are significantly different ($P < 0.01$), $n = 6$.

[1] APC = aerobic plate count, colony forming units per mL ($\log_{10}$).

[2] Control = after cheesecloth screening: ozonation = 5 min, 1.37% ozone; filtration = one pass through a slow sand filter.

Significant reductions in levels of COD, TS, FOG, APC, COLIF, *E. coli*, and *Salmonella* of 87, 65, 95, 99.9, 99.8, 99.7, and 99.9%, respectively, were detected after filtration and ozonation (DE/O_3, Table 2.1.19). The %T averaged 97% following this treatment and easily met the recycling criteria, as did the microbial reductions (greater than 99.9% reduction).

In this study DE filtration with a filter designed for swimming pools was not effective in reducing the chiller water microbial contamination loads. A final disinfection step with ozone yielded water free of COLIF and SALM.

Two other process waters, final carcass rinse and neck chiller overflow waters, also were successfully reconditioned with DE filtration and ozone. Because neck chiller overflow water (Table 2.1.20) had considerably higher organic loads than the final carcass rinse or chiller waters, it was recirculated through the DE filter for 10 min. This water treatment was also very effective in improving the water quality of these two process waters and satisfied the USDA chiller water recycling criteria. Significant reductions in levels of COD (78%), TS (51%), APC (96%), COLIF (99.5%), *E. coli* (99.5%) and SALM (99.9%) were obtained in comparison with those of Control 2, whereas %T increased to 80.1% from 1.7% following this treatment. This reconditioned water would qualify for recycling under the USDA chiller water regulations at a rate of 1. 1 gallon (4.16 L) of reconditioned water to replace every gallon (3.78 L) of fresh water.

Table 2.1.19. Effect of diatomaceous earth (DE) filtration in combination with ozonation on the quality of reconditioned broiler overflow prechiller water [1] (Chang and Sheldon, 1989a)

Treatment [2]	**APC, CFU/mL**	**Coliforms, CFU/mL**	***E. coli*, MPN/mL**	***Salmonella*, MPN/mL**
Control 1 [3]	3.9 [A]	----	----	----
Control 2	4.0 [A]	3.0 [A]	2.8 [A]	> 1.4 [A]
DE [3]	4.5 [B]	----	----	----
DE/O_3	0.5 [B]	< 0.03 [B]	< 0.3 [B]	< -1.5 [B]

[A,B] Means within the same column with no common superscripts are significantly different ($P < 0.01$), n = 6.

[1] APC = aerobic plate count, colony forming units per mL ($\log_{10}$); coliform bacteria, most probable number (MPN/mL) ($\log_{10}$).

[2] Control 1 = before treatment; Control 2 = after cheesecloth screening, DE = 5 min filtration, DE/O_3 = 5 min filtration, 15 min ozonation (2.30% ozone).

[3] No data for measures of coliforms, *E. coli* and *Salmonella*.

Table 2.1.20. Effect of diatomaceous earth (DE) filtration in combination with ozonation on the quality of reconditioned broiler neck chiller overflow water [1] (Chang and Sheldon, 1989a)

Treatment [2]	APC, cfu/mL	Coliforms, cfu/mL	*E. coli*, MPN/mL	*Salmonella*, MPN/mL
Control 1 [3]	4.4 [A]	----	----	----
Control 2	4.4 [A]	2.6 [A]	2.6 [A]	> 0.4 [A]
DE/O_3	1.0 [B]	< 0.3 [B]	< 0.3 [B]	< -2.5 [B]

[A,B] Means within the same column with different superscripts are significantly different ($P < 0.01$), n = 6.

[1] APC = aerobic plate count, colony forming units per mL (log_{10}); coliform bacteria, most probable number (MPN/mL) (log_{10});

[2] Control 1 = before cheesecloth screening; Control 2 = after cheesecloth screening, DE/O_3 = DE filtration for 10 min/ozonation for 15 min (2.43% ozone).

[3] No data for measures of coliforms, *E. coli* and *Salmonella*.

Final carcass rinse water contained the lowest amount of contaminants of all the waters examined in this study. Significant reductions in levels of COD (89%), TS (54%), APC (>99.9%), COLIF (99.9%), *E. coli* (99.9%), and SALM (99.9%) were obtained in comparison to screened samples (Control 2) following treatment (Table 2.1.21). The COLIF, *E. coli* and SALM were not detected in the reconditioned final carcass rinse water after ozonation.

Table 2.1.21. Effect of diatomaceous earth (DE) filtration in combination with ozonation on the quality of reconditioned final carcass rinse water [1] (Chang and Sheldon, 1989a)

Treatment [2]	APC, CFU/mL	Coliforms, CFU/mL	*E. coli*, MPN/mL	*Salmonella*, MPN/mL
Control 1 [3]	4.8 [A]	----	----	----
Control 2	4.8 [A]	2.3 [A]	2.3 [A]	> 0.4 [A]
DE/O_3 [4]	0 est [B]	< 0.3 [B]	< 0.3 [B]	< -2.5 [B]

[A-C] Means within the same column with different superscripts are significantly different ($P < 0.01$), n = 6.

[1] APC = aerobic plate count, colony forming units per mL (log_{10}); coliform bacteria, most probable number (MPN/mL) (log_{10});

[2] Control 1 = before cheesecloth screening; Control 2 = after cheesecloth screening, DE/O_3 = DE filtration for 5 min/ozonation for 15 min (2.54% ozone).

[3] No data available for coliforms, *E. coli* and *Salmonella*.

[4] 0 est = No culturable microorganisms detected.

Of all the treatments tested, a combination of screening, DE filtration, and ozonation yielded the highest quality water. After treatment the reconditioned water qualified to be recycled back to the chiller at a rate of 1.1 gal of reconditioned water to replace every 1 gal of fresh water.

2.1.2.8 Chang and Sheldon (1989b) – Broiler Prechiller Water

Broiler prechiller overflow water was reconditioned using diatomaceous earth (DE) filtration and ozonation (30.4 mg/L ozone). Reconditioned chiller water meeting the USDA recycling criteria was used in chilling broiler carcasses. The carcasses were compared chilled with fresh water and crushed ice or with reconditioned chiller water and ice. No significant treatment differences between measures of carcass quality including skin color, taste, or shelf life were detected. Furthermore, no significant differences between whole carcass rinse aerobic plate counts, or coliform and *Salmonella* counts were found for carcasses chilled with fresh or reconditioned chiller water. Poultry processing waters may be conserved through recycling practices without adversely affecting the wholesomeness of the carcass.

Experimental procedures were similar to those reported in earlier publications by Prof. Sheldon and his colleagues and listed in this petition.

Experimental Design. Approximately 160 L of prechiller overflow water from a commercial poultry processing plant were initially screened through five layers of cheesecloth and then recirculated through a presanitized DE filter for 5 min at a flow rate of 159 L/min. The filter flextubes were precoated with 2.25 kg of Celatom diatomite. Following filtration, the water was ozonated for 15 min in a presanitized sparge tower described below (30.4 mg/L ozone, 86 L/min water flow rate). Water was sampled and evaluated after screening and ozonation for light transmission and microbiological characteristics to determine if it satisfied the USDA recycling criteria before proceeding with the carcass chiller studies.

Both reconditioned chiller water and fresh water were chilled overnight at 4.4EC in covered, presanitized stainless steel (SS) vats. Twelve unchilled broiler carcasses per treatment were chilled for 30 min in 160 L of either reconditioned water or potable water (control) in a 190-L, presanitized stainless steel vat. Approximately 22.5 kg crushed ice/treatment was added to the 160 L of water to maintain a water temperature of about 2EC. Vat contents were agitated using a Heidolph Model R2R30 stirrer, 46 rpm. The stirrer shaft was attached to a square 19.5-cm Plexiglas plate having nine 1.5-cm diam holes evenly distributed over the plate. The carcass temperatures following chilling were 2 to 3EC. After chilling the carcasses were hung on shackles and drained for 15 min in a 4.4EC cooler, individually weighed for estimating surface area, and packed in oxygen-permeable plastic bags. All carcasses were stored at 4.4EC with two carcasses per treatment sampled at 0, 4, 7, 9, 11, and 13 days to determine whole carcass rinse total aerobic plate counts (APC and psychrotrophic plate counts (PPC).

The CIE (International Commission on Illumination) L*, a*, and b* skin color reflectance values of the back, breast, and drumstick; whole carcass rinse coliform (COLIF), *E. coli*, and *Salmonella* (SALM) counts; and sensory evaluations also were determined on two carcasses/treatment on Day 0. Two hot, nonchilled broiler carcasses were evaluated for skin color and microbial loads for comparison with chilled carcasses. All filtration and ozonation equipment used in this experiment was presanitized with 10% chlorox for 15 to 20 min then rinsed with potable water for 15 to 20 min prior to each run. This experiment was replicated three times using reconditioned chiller water sampled on three separate days.

Ozone Contact System. An ozone contact sparge tower was constructed using a 3.35-m polyvinyl chloride (PVC) pipe (15.24 cm i.d.). The water level was maintained at 3.05 in and monitored using a clear sight tube height indicator attached to the side of the column. Approximately 160 L of water was pumped at 86 L/min from a 190-L stainless steel holding reservoir to the top of the column using a centrifugal pump with a sanitary relief valve for throttling the flow rate. Water exited from the bottom of the column into the holding reservoir and was recirculated back to the contractor for 15 min. Nonsolubilized gases exiting the top of the column were not recycled. Ozone entered the bottom of the column through a 2-g stainless steel porous diffuser countercurrent to the water flow.

Broiler Sample Collection. Hot broiler carcasses were sampled approximately 2 h after plant startup from a local commercial poultry processing plant just as they were to enter the prechiller. Carcasses were held in water-impermeable plastic bags under ice (without direct contact) while being transported to the lab. Treatment began within 30 min of sampling.

Bacteriological Methods. Individual broiler carcasses were rinsed in plastic bags with 100 mL of sterile distilled water using a whole bird rinse technique. The water rinses for two carcasses were combined, the volume measured (approximately 150 mL) and then serially diluted in 0.1% peptone water. Aerobic plate counts (32EC, 48 h) and psychrotrophic counts (7EC, 10 days) were determined using Plate Count Agar. Total coliforms and *E. coli* were enumerated by a five-tube most probable number (MPN) technique as described by Sheldon and Brown (1986). All counts were reported on a basis of volume per milliliter of recovered rinse water and can be multiplied by the total volume of rinse water recovered (average of 75 mL/carcass) to calculate the total count per volume of rinse water. Isolation and enumeration of *Salmonella* from each carcass was based on a three-tube MPN technique as described by Sheldon and Brown (1986) using a 10, 1, and .1-mL rinse water sample size. *Salmonella* counts were reported per milliliter of rinse water. Aerobic plate counts (32EC, 48 h) were determined on reconditioned water using standard plate count methods.

RESULTS AND DISCUSSION

Quality characteristics of the reconditioned prechiller overflow water are presented in Table 2.1.22. The aerobic plate count was reduced by an average of 2.7 logs or 99.6% following treatment. Based on the significant reductions noted in previous studies, 99.9% for coliforms and 98% for *Salmonella,* coliforms and *Salmonellae* microorganisms were not evaluated. Light transmission properties of the reconditioned water were significantly improved from an average of 19.4% before treatment to 95.7% after treatment. Based on these analyses the reconditioned water would qualify for recycling to the chiller at a rate of 1.1 gal (4.16 L) of reconditioned water to replace every gallon (3.78 L) of fresh water. The pH of the reconditioned water decreased an average of 0.3 pH units, indicating the oxidative destruction of lipid and protein by ozone.

The microflora, shelf life, skin color, and flavor of potable water-chilled and recycled water-chilled carcasses were compared. There were no significant differences between carcasses chilled with potable water and those chilled with reconditioned water for APC, PPC, COLIF, *E. coli*, and SALM (Table 2.1.23). No significant replicate differences were noted within each treatment for each of the individual carcass counts.

Individually packaged carcasses were stored at 4.4EC to evaluate shelf life differences of carcasses chilled with potable water or reconditioned chiller water (Table 2.1.24). No significant treatment differences in APC or PPC were detected over 13 days of refrigeration, for carcasses chilled in reconditioned water or potable water. As expected, PPC increased at a higher rate than APC during refrigeration. Furthermore, neither the potable water nor reconditioned water chilled carcasses showed evidence of spoilage (off-odors, slime) at the end of 13 days of refrigeration. In general, refrigerated poultry carcasses are contaminated with both pigmented and nonpigmented strains of *Pseudomonas* and *Acinetobacter*. Using a conversion factor of 1,900 cm^2 for a 2.5 lb (1,135 g) broiler, the 13-day geometric mean (log_{10}) aerobic and psychrotrophic plate counts would be 7.19 and 7.88/cm^2, respectively, for carcasses chilled with potable water vs. 7.17 and 7.75, respectively, for carcasses chilled with reconditioned chiller water. Broilers used in this study ranged in weight from 1,050 to 1,500 g.

Table 2.1.22. Quality of reconditioned chiller overflow water following diatomaceous earth (DE) filtration and ozonation [1] (Chang and Sheldon, 1989b)

Treatment [2]	Replicate	APC, CFU/mL
Control	1	4.3
	2	4.1
	3	4.8
	⌋ (n = 3)	4.4
DE/O_3	1	1.0
	2	2.1
	3	2.0
	⌋ (n = 3)	1.7

[1] APC = aerobic plate count, colony forming units per mL (log_{10}, geometric mean) after 24 h storage at 4.4EC.

[2] Control: after cheesecloth screening; DE/O_3: 5 min DE filtration/15 min ozonation.

Table 2.1.23. Effect of chilling broiler carcasses with reconditioned prechiller overflow water on carcass microflora [1] (Chang and Sheldon, 1989b)

Treatment [2]	APC, CFU/mL	PPC, CFU/mL	Coliform [3] MPN/mL	*E. coli* MPN/mL	*Salmonella* MPN/mL
Hot	5.05	4.15	4.13	4.03	< 1.38
Fresh water	4.73	4.33	3.38	3.55	< 1.38
Reconditioned water	4.93	3.95	3.62	3.55	< 1.38

[1] There were no significant differences among treatments; n = 3.

[2] Hot = unchilled carcasses; fresh water = carcasses chilled in fresh potable water; reconditioned water = carcasses chilled in reconditioned chiller overflow water (geometric mean, per mL of carcass rinse, log_{10}, mean times 75 mL = mean count per total carcass time, three replicates).

[3] MPN = Most probable number.

Table 2.1.24. Effect of chilling broiler carcasses with reconditioned prechiller overflow water on the shelf life of broilers stored at 4.4EC [1] (Chang and Sheldon, 1989b)

Day	Control		Reconditioned	
	APC [2]	PPC [3]	APC	PPC
0	4.7 [C]	4.3 [E]	4.9 [C]	4.0 [D]
4	5.2 [C]	5.7 [D]	5.4 [C]	6.0 [C]
7	7.0 [B]	7.6 [C]	7.4 [B]	7.9 [B]
9	7.8 [AB]	8.5 [B]	8.0 [AB]	8.5 [AB]
11	8.2 [A]	9.1 [AB]	8.2 [AB]	8.9 [A]
13	8.6 [A]	9.3 [A]	8.6 [A]	9.2 [A]

[A-E] Means within the same treatment and bacteria type with no common superscripts are significantly different (P < 01). There were no significant differences between treatments within day and bacteria type.

[1] Geometric means of three experimental replicates (two carcasses per sampling); control = carcasses chilled with fresh potable water; reconditioned = carcasses chilled with reconditioned chiller overflow water.

[2] Aerobic plate count log,,,, colony-forming units per milliliter of carcass rinse, mean times 75 mL = mean count per total carcass rinse.

[3] Psychrotrophic plate count $\log_{10}$, colony -forming units per milliliter of carcass rinse, mean times 75 mL = mean count per total carcass rinse.

2.1.2.9 Izat et al. (1990) – Giblet Chiller Water

Eviscerated broiler carcasses were chilled in a 2-sided rotary giblet chiller, with 200 randomly selected carcasses assigned to a control side and 200 assigned to an ozone-treated side. Chlorine was added to the control side at 20 ppm which is standard practice in the industry. Water temperature in the chiller was maintained at 1.7-4.4EC by addition of crushed ice. Oxidation-reduction potential (ORP) of the control chill water began at 900 mV on a scale of 0 to 1000. As carcasses were introduced into the chiller the ORP level fell, resulting in an average of 270 mV over the entire period. **The ozone generator utilized produced 20 g of ozone per hour, sufficient to maintain only 300-400 mV in the chill water.**

Water samples were collected in sterile bottles from each side of the chiller tank after 0, 20, 50, 100, 120, 140, 160, 180, and 200 carcasses had been chilled. Samples also were taken from the ozonated makeup (recycled) water after 140, 160, 180, and 200 carcasses had been chilled. Each sample was evaluated for incidence of *Salmonellae*, levels of total organisms, levels of presumptive coliforms, and percent light transmittance at 500 nm. Data were analyzed statistically using the General Linear Model procedure and means separated using Least Square Means.

Microbiological Quality: *Salmonellae* were detected at various times in both the control and ozonated chill water (see Table 2.1.25). No *Salmonellae* were detected in any of the ozonated

makeup water samples. After the first 20 carcasses were chilled, levels of total organisms and presumptive coliforms were significantly higher in the ozonated side of the chiller than in the control side. **This suggests that either insufficient ozone was generated or the ozone was not remaining in solution for a sufficient time to affect bacterial numbers.** The dramatic reduction in levels of total organisms and presumptive coliforms in the ozonated makeup water as compared to water in the ozonated chill tank suggests that this relatively small amount of recycled water was being treated effectively. In all samples there was a 69 to 99% reduction in total organisms and presumptive coliforms, meeting the current USDA requirements for water reuse.

Table 2.1.25. Effects of ozonation on microbiology and clarity of processing waters (Izat et al., 1990)

Parameter	Number of carcasses chilled								
	0	**20**	**50**	**100**	**120**	**140**	**160**	**180**	**200**
Salmonella indicence[1]									
Control chiller [2]	nd	Nd	nd	+	nd	nd	+	nd	+
Ozonated chiller	nd	nd	+	nd	nd	nd	+	nd	+
Ozonated makeup [3]	na	na	na	na	na	nd	nd	nd	nd
Light transmittance [4]									
Control chiller	100a	97a	90.5a	88b	90b	86a	81.5a	82a	84.5a
Ozonated chiller	100a	98a	89a	84a	80b	85.5a	81a	82.5a	84.5a
Ozonated makeup	na	na	na	na	na	93b	91.5b	93.5b	89.5b
Log_{10} APC[5]/mL									
Control chiller	1.54a	1.54a	1.50a	1.15a	1.67a	1.85a	3.86a	4.05b	4.57b
Ozonated chiller	1.54a	2.93b	6.48b	3.58b	4.02b	6.47b	4.54c	4.72b	4.69b
Ozonated makeup	na	na	na	na	na	0.18a	4.08b	1.57a	3.00a
Log_{10} coliforms/mL									
Control chiller	-0.30a	-0.30a	-0.30a	-0.30a	-0.30a	-0.45a	-0.30a	2.70b	1.60a
Ozonated chiller	-0.30a	1.59b	1.29b	2.68b	2.64b	1.77b	1.80b	2.90b	2.86b
Ozonated makeup	na	na	na	na	na	-0.30a	-0.30a	-0.30a	-0.30a

[1] nd = *Salmonellae* not detected, lower detection level = 0.03 CFU/mL. [2] na = not assayed. [3] Control chill water was chlorinated at 20 ppm. [4] Percent transmission at 500 nm. [5] APC = Aerobic plate count.

Comment by Petitioner: Even though significant reductions in microorganism levels were observed, there was insufficient ozone generated by the equipment employed to attain even better reductions. This point was made clearly by the authors of this article.

2.1.2.10 Waldroup et al. (1993) – Recycling Chiller Water

Summary: A study was conducted to determine whether ozone could be used to meet USDA requirements for recycling poultry chill water. The system as approved by USDA used an ozone dose rate of 7.0 to 11.7 ppm in a series of four 580-gallon tanks. Total contact time during ozonation was approximately30 minutes. Reductions in excess of 99% were achieved for total aerobes, *E. coli*, and presumptive coliforms. After ozone treatment, light transmission ranged from 88 to 99% (540 nm) as compared to fresh water. USDA approval of the system was granted in early spring of 1991.

Ozonation System: A prototype water recycling ozonation system was installed and evaluated in a commercial turkey poultry processing facility over a 4-month period. The ozonation system consisted of a hydrasieve followed by a separation tank at the overflow end of the chill tank. Both the hydrasieve and separation tank functioned to remove a percentage of solids from the chill water prior to ozonation. Water exiting the separation tank was pumped outside the plant to the ozonation system. Ozonation occurred in a series of four vertical stainless steel tanks (580 gallons/tank). Chill water entered through the top of each interconnected tank, while an ozone:air mixture was injected at the bottom.

Injection of ozone was accomplished by two methods. In tanks 1 and 2, water from the bottom of each tank was pumped through separate venturi injectors where ozone was injected. Static mixers in each tank were used to return the ozone:water mixture to the chill water column in the bottom of each tank. In tanks 3 and 4, ceramic diffusers dispersed ozone into the chill water. The tanks were closed systems with a collection funnel at the top of each where flocculant composed primarily of suspended fats and proteins was removed for rendering.

During normal operation, the ozone application rate was 7.0, 9.4, 9.4, and 11.7 ppm for tanks 1, 2,3, and 4, respectively. An ozone monitor allowed computer control of water flow rates and ozone levels in the four tanks during operation. The system was capable of processing 80 gallons of chill water per minute with an average water loss of 10% during normal operation. During the evaluation periods all ozone-treated water was discharged as waste.

Microbiological Sampling: Water samples were collected and evaluated on October 7 through 10, 1990 and on January 9, 10, 11, 14, and 15, 1991. Duplicate samples were collected at 0800, 1000, 1200, and 1400 hours on each sampling day. The plant had been operating for at least two hours when sample collection was initiated.

Untreated (control) samples were collected directly from the overflow of the chill tank before the hydrasieve. Treated samples were collected at the exit of the fourth ozonation tower and after charcoal filtration (in January, 1991 only). Immediately following collection, the samples were placed in an insulated container and held at 34E to 36EF until assays were initiated. The time between sample collection and microbial evaluation did not exceed six hours. All samples were evaluated in duplicate for enumeration of aerobic organisms (APC), presumptive coliforms, and *E. coli* using Petrifilm™.

RESULTS AND DISCUSSION

Microbiological Analyses: Neither *E. coli* nor total coliforms could be detected after ozonation at any sampling time on any of the four days. Levels in the pretreatment chill water ranged from $\log_{10}$ 0.68 to 1.28 CFU/mL and $\log_{10}$ 0.51 to 1.39 CFU/mL for *E. coli* and total coliforms, respectively. Reductions met USDA recycling specifications and allowed for maximum recycling rates (90% of chill water).

As Table 2.1.26 shows, no aerobic bacteria were detected after ozonation on any day at the first three sampling times (0800 to 1200 hours). However, low levels of aerobes were detected after ozonation at 1400 hours on each of the four days. Incomplete destruction of organisms at 1400 hours could be due to higher levels of organisms in the chill water at that time of day, or this

finding might have resulted from a change in carcass-to-water ratio. It is known that bacterial counts in chill water increase during the processing day. Light transmission values at 1400 hours on all four days indicate a greater than normal percentage of organic matter in the post-ozonation samples.

Table 2.1.26. Effect of ozonation on $\log_{10}$ aerobic organisms (CFU/mL) in poultry chill water (Oct. 1990) (Waldroup et al., 1993)

Date	Location [A]	Sampling Time (hours)				
		0800	1000	1200	1400	X BAR
10-07-90	1	2.10^{c}	2.50^{b}	2.95^{a}	2.90^{a}	2.64
	2	ND^{e}	ND^{e}	ND^{e}	0.35^{d}	0.09
	SEM=0.05					
10-08-90	1	2.11^{c}	2.66^{b}	2.88^{a}	2.81^{a}	2.61
	2	ND^{e}	ND^{e}	ND^{e}	0.15^{d}	0.04
	SEM=0.04					
10-09-90	1	2.20^{c}	2.42^{bc}	2.46^{b}	2.94^{a}	2.50
	2	ND^{e}	ND^{e}	ND^{e}	0.51^{d}	0.13
	SEM=0.09					
10-10-90	1	2.10^{d}	2.22^{c}	2.59^{b}	2.83^{a}	2.44
	2	ND^{f}	ND^{f}	ND^{f}	1.02^{e}	0.26
	SEM=0.03					

[A] Location 1 and 2 correspond to overflow from the chiller and post-ozonation treatment, respectively.

[a-f] Interaction means (n = 4) with the same superscript do not differ significantly (P # 0.05).

ND = aerobic organisms not detected; lower detection level (LDL) = 1.0 CFU/mL.

Microbiological Analyses: Levels of *E. coli* and presumptive coliforms in the pretreatment samples ranged from $\log_{10}$ 1.2 to 1.5 CFU/mL and $\log_{10}$ 1.1 to 1.6 CFU/mL, respectively. No *E. coli* or presumptive coliforms were detected in any post-ozonation or post-charcoal filtration samples. Reductions allowed for maximum water recycling (90% of chill water) as specified by the USDA.

On each of the five sampling days there was a significant time of day/by location of sampling effect on APCs (Table 2.1.27, inadvertently omitted). At every sampling time post-ozonation treatment and post-charcoal filtration samples showed significant reductions in levels of APCs compared to pretreatment samples. In several instances APCs increased significantly from post-ozonation to post-charcoal filtration, indicating that contamination occurred during charcoal filtration. However, post-ozonation and post-charcoal filtration APC levels were consistently low during all five of these days and met USDA recycling specifications while allowing for maximum water recycling. The manufacturer of the ozonation system presently advises the user to allow the charcoal filtration system to remain in a household oven at 500EF for eight to ten

hours at the end of each processing day. This procedure allows for thorough disinfection of the filtration unit.

Table 2.1.27. (inadvertently omitted)

All data collected during this five-day sampling period in January 1991 - including light transmission, microbial reductions, and TOC determinations - were used as the final criteria to obtain USDA approval for the AWPI system for recycling poultry chill water. Approval of the recycling system was granted in early spring of 1991.

2.1.2.11 Jindal et al. (1995) – Chiller Water

Summary: Three experiments attempted to evaluate the efficacy of using ozone during immersion chilling for improving the microbial safety and extending the shelf life of broiler drumsticks. Pre-chill drumsticks were treated with ozone (0.44 to 0.54 ppm) or air during immersion chilling (45 min at 0 to 4EC) and then individually wrapped and stored at 1 to 3EC. Ozone reduced the levels of aerobic plate count, coliforms, and *E. coli* on broiler drumsticks by more than 1.11, 0.91, and 0.90 logs, respectively. Levels of *Pseudomonas aeruginosa*, Gram-negative, and Gram-positive bacteria were reduced by 038, 1.11, and 1.14 logs in the third experiment. Ozonation extended the shelf life (product was considered spoiled at $\exists \log_{10}$ 7.0 CFU/cm^2) of broiler drumsticks for as much as two days. Reductions in microbial levels noted in poultry chill water were even greater than those on the surface of drumsticks. Levels of bacteria declined by 135 to 2.82 logs (95.5 to 99.8%).

Ozone Chilling System: Ozone was generated by passing cold air through ultraviolet lamp chambers in two ozone generators. A prototype poultry chill tank with a capacity of 130 L chilled the test product. An exhaust system containing activated charcoal and an exhaust fan minimized the release of ozone from the chill tank. Ozone was dispersed in the chill water at five water inlets, with water continually recirculating in the chill tank by means of a water pump operating at 456 L/min. The flow of ozone was controlled by an injector manifold with five injector valves. A stainless steel cylinder placed inside the chill tank provided circular movement of broiler drumsticks during chilling. An air supply mechanism attached to an accumulator tank provided ozone-free air for treating control drumsticks. In these experiments chlorine was not added to the tap water; thus, an initial chlorine concentration of < 1 ppm was present in all the water utilized for chilling. A copper heat exchanger was attached to the incoming water supply to maintain chill water temperature at < 4.0EC. The heat exchange coil was placed in a vessel filled with crushed ice and cold water. All variables, except the use of ozone, were held constant for control (air) and ozone treatments during the chilling procedure. In each experiment, the prototype ozonation. system provided 60 mg/min of ozone.

Microbiological Analyses: Broiler drumsticks and chill water samples were analyzed for aerobic plate count (APC), *E. coli*, and coliforms in Experiments 1 and 2. In addition to these

groups of microorganisms, *Pseudomonas aeruginosa* and total Gram-negative bacteria also were enumerated on drumsticks and chill water samples in Experiment 3. Total Gram-positive organisms were calculated by subtracting the value for Gram-negative bacteria from APC.

Drumsticks were sampled immediately after chill water treatment using a whole carcass rinse procedure. Drumsticks were placed in sterile plastic bags and rinsed with 25 mL 0.1% peptone water and shaken with an automatic shaking device for 1 min. Chill water samples (100 mL) were collected in sterile glass bottles at the end of the chilling period in Experiments 1 and 2. In Experiment 3, water samples were analyzed at time 0 (before adding prechill drumsticks), and after 15, 30, and 45 min of chilling. All samples were stored in crushed ice prior to microbial analyses. The APC, *E. coli*, and coliforms were enumerated using Petrifilm. APC plates were incubated at 30EC for 48 hr; and *E. coli* plates were incubated for 24 hr at 350EC. Total Gram-negative bacteria were enumerated on McConkey agar by incubating for 12 hr at 35EC; and *Pseudomonas aeruginosa* were enumerated on a Cetrimide agar base by incubating for 24 hr at 35EC.

Experimental Design: Numerous preliminary experiments were conducted to evaluate the efficacy of ozone in reducing the levels of bacteria on whole carcasses and on cut-up broiler parts. Three shelf life experiments were conducted. In each experiment, 100 drumsticks were harvested from pre-chill carcasses processed at the University of Arkansas pilot poultry processing facility. Twenty-five broilers were removed from the processing line and 50 drumsticks were harvested. Fifteen min after the drumsticks were harvested, they were treated with air injected into the chill water for 45 min at #4EC. A second group of 50 drumsticks was treated with ozone in a process similar to the air treatment. In each of three experiments, 22.5 kg of ice was placed in the prototype chill tank; the chill tank was then filled with 100 L cold water (6.0EC). The heat exchange unit maintained the water at #4EC.

Five drumsticks and two chill water samples (100 mL) for each treatment were analyzed microbiologically on day 0 in Experiments 1 and 2. Two water samples for each treatment time were analyzed at time 0 (before adding prechill drumsticks), and at 15, 30, and 45 min after adding the drumsticks in Experiment 3. The remaining 45 drumsticks in each air or ozone treatment were individually wrapped with plastic film and were stored at 1 to 3EC. Five drumsticks from each treatment were selected randomly on days 2, 4, 7, 9, 11, and 12 in Experiment 1; on days 2, 4, 6, 8, 10, and 11 in Experiment 2; and on days 2, 4, 6, 8, 10, 11, 12,13, and 14 in Experiment 3. All data were subjected to statistical analyses to determine chill water treatment effects.

RESULTS AND DISCUSSION

Ozonation Effects on APC Levels and Shelf life of Drumsticks: In all three experiments, the interaction effect (days of storage by treatment) was significant for all groups of microorganisms. The APC levels were consistently lower on the ozone-treated drumsticks as compared to levels on air-treated drumsticks in all three experiments (Figures 2.1.09, 2.1.10, and 2.1.11). Except on days 7 and 12 in Experiment 1, the difference in APC levels was significant between the two treatment groups. On day 0, ozone significantly reduced APC levels by 1.30, 1.25, and 1.11 logs in Experiments 1, 2, and 3, respectively. However, the difference in APC levels between the two treatment groups was reduced to 0.30 and 0.45 log in Experiments 1 and 3 at the end of the respective storage periods. In Experiment 2, levels of APC were not enumerated for the air treatment after the drumsticks were spoiled. Microbial growth during refrigerated storage increased levels of APC on both the air- and ozone-treated drumsticks, resulting in less of a difference in APC levels between the two treatment groups.

Poultry meat is considered spoiled at $\log_{10}$ 7.0/cm^2. Using this criterion, the air-treated drumsticks spoiled between 9 and 13 days in all three experiments (Figures 2.1.09, 2.1.10, and 2.1.11). The ozone-treated drumsticks spoiled on day 12 (Experiment 1) and day 13 (Experiment 2). In Experiment 3, the ozone-treated drumsticks did not spoil even after 14 days of refrigerated storage. Thus, ozonation extended the shelf life of broiler drumsticks at 3EC for at least one and usually for two days. Drumsticks containing higher initial levels of APC, as in Experiment 1, spoiled earlier than those having lower initial levels of APC (Experiments 2 and 3) for both the air and ozone treatment groups.

Results from the present study demonstrate that ozonation can extend the shelf life of broiler drumsticks by at least one day. The differences in shelf life extension as reported in the literature probably result from the particular ozonation system utilized and concentration of ozone in the chill water.

Ozonation Effects on Coliforms, *E. coli, Pseudomonas aeruginosa*, Gram-negative, and Gram-positive Bacteria: Levels of coliforms on ozone-treated drumsticks remained significantly lower than levels on air-treated drumsticks throughout the shelf life study in Experiment 1 (Figure 2.1.09). In Experiments 2 and 3, levels of coliforms were significantly lower until day 10 (Figures 2.1.10 and 2.1.11). Coliform levels decreased during storage for both groups of drumsticks. However, the difference in coliform levels between the air and ozone treatments decreased with increased storage.

Similarly, the difference in *E. coli* counts between the two treatment groups decreased continuously during refrigerated storage (Figure 2.1.09). On day 0, *E. coli* counts were significantly lower on the ozone-treated drumsticks by 1.29, 1.00, and 0.90 logs in Experiments 1, 2, and 3 (Figures 2.1.09, 2.1.10, and 2.1.11). *E. coli* were not detected at the end of refrigerated storage for either group of drumsticks. During storage, *E. coli* on ozone-treated drumsticks were eliminated earlier (days 6 and 10) than on air-treated drumsticks (days 11 and 12) in Experiments 2 and 3.

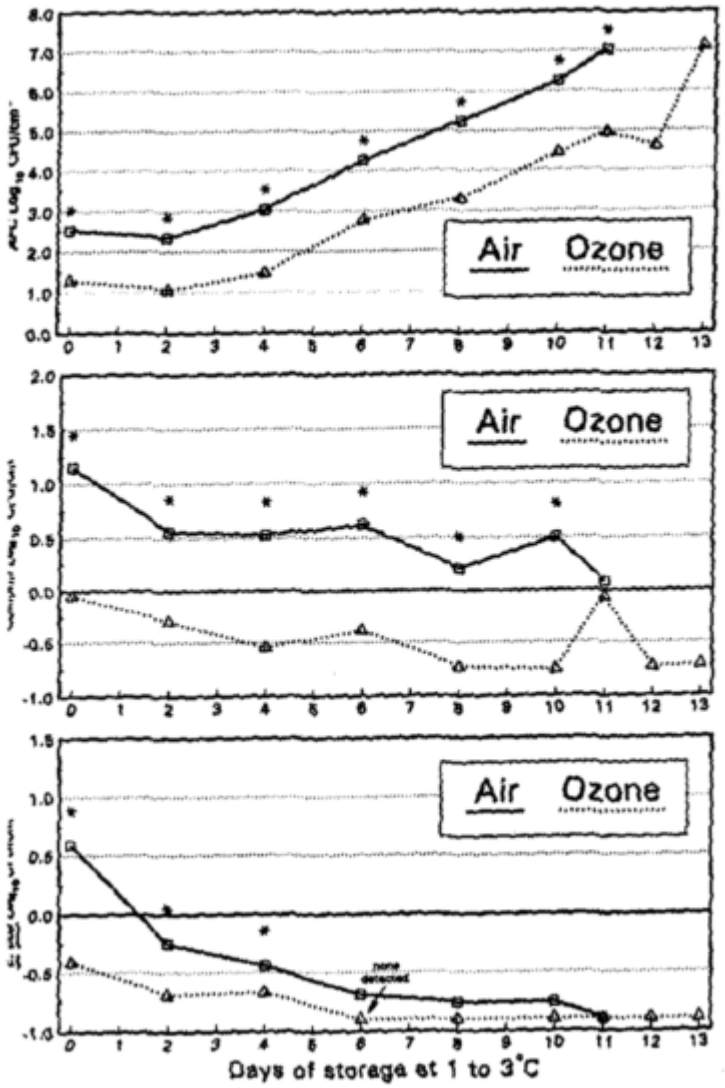


Figure 2.1.09. Effects of ozonation during immersion chilling on the microbiological quality (APC, coliforms and *E. coli*) of broiler drumsticks (n = 5) during refrigerated storage, Experiment 1. Within days of storage, an asterisk indicates a significant difference between treatments (Jindal et al., 1995).

In Experiment 3, *Pseudomonas aeruginosa*, Gram-positive, and Gram-negative bacteria also were enumerated on broiler drumsticks (Figure 2.1.12). On day 0, ozonation reduced the levels of *Pseudomonas aeruginosa*, Gram-negative, and Gram-positive bacteria by 0.38, 1.11, and 1.14 logs, respectively. Levels of these three bacteria increased throughout the refrigerated storage period on both the air- and ozone-treated drumsticks, except on day 14. *Pseudomonas aeruginosa* and Gram-negative bacteria counts on ozone-treated drumsticks decreased on day 14

as compared to respective counts on day 13. Ozone-treated drumsticks contained significantly lower counts of *Pseudomonas aeruginosa* and Gram-negative bacteria as compared to air-treated drumsticks until day 12. Ozonation significantly reduced the Gram-positive bacteria throughout refrigerated storage.

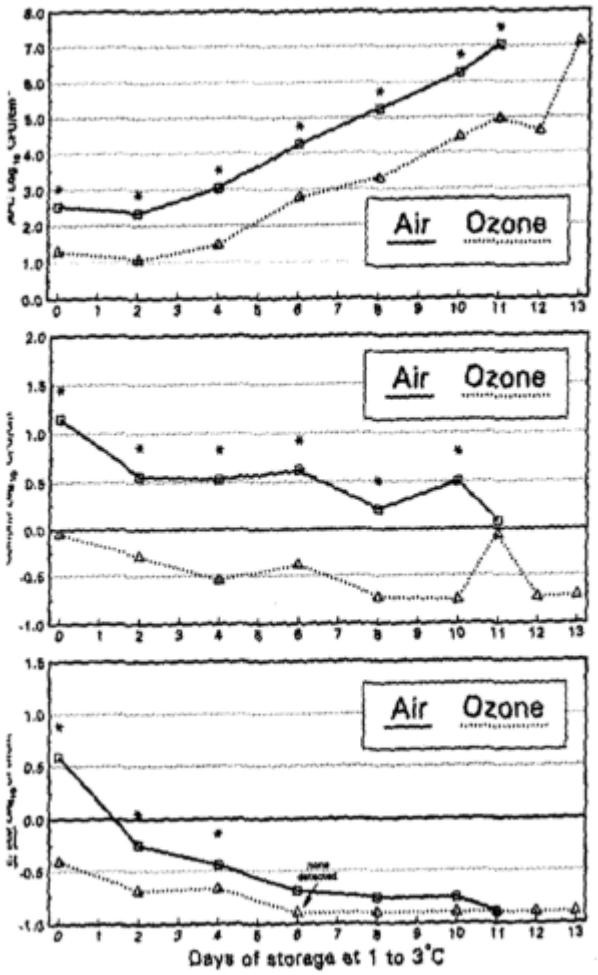


Figure 2.1.10. Effects of ozonation during immersion chilling on the microbiological quality (APC, coliforms and *E. coli*) of broiler drumsticks (n = 5) during refrigerated storage, Experiment 2. Within days of storage, an asterisk indicates a significant difference between treatments (Jindal et al., 1995).

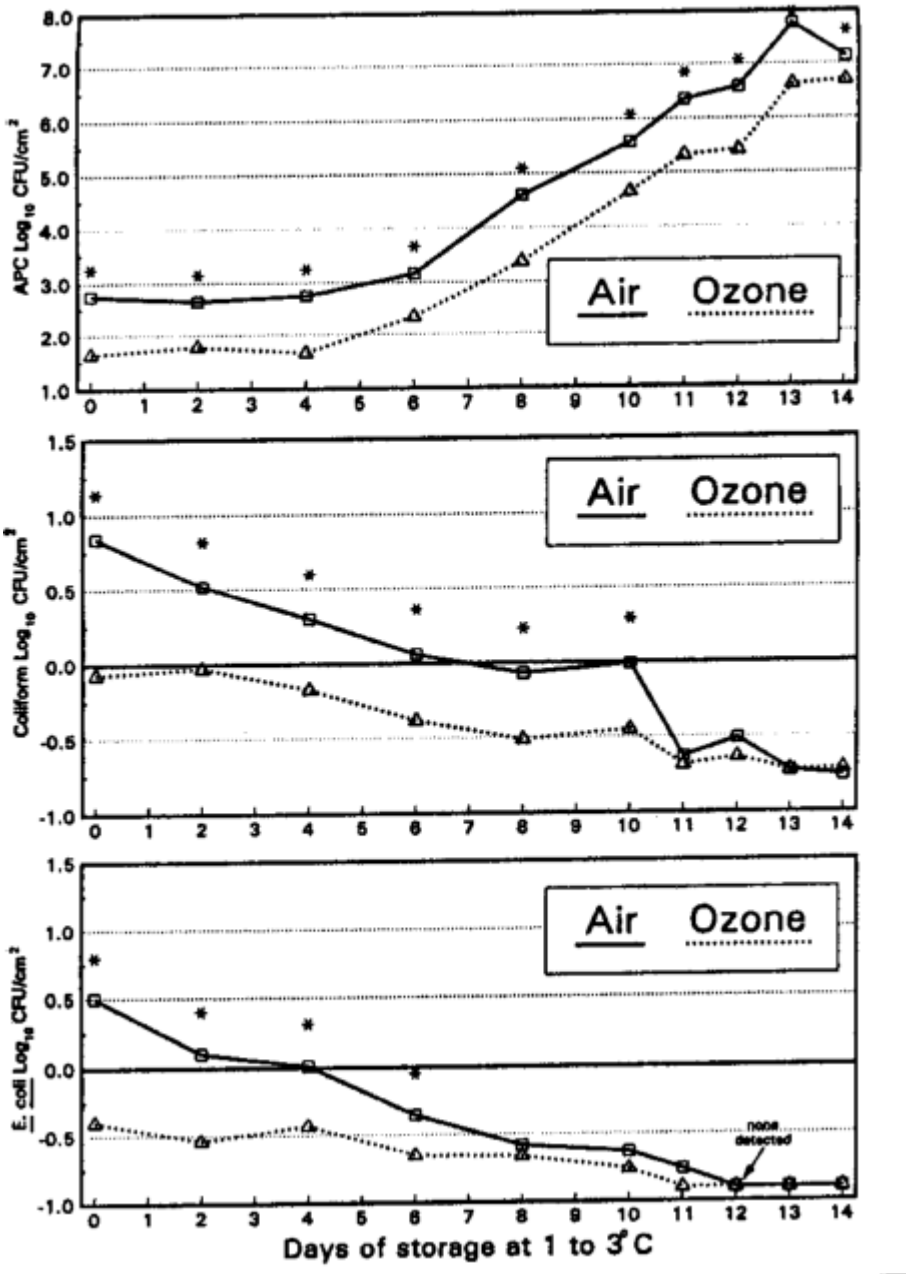


Figure 2.1.11. Effects of ozonation during immersion chilling on the microbiological quality (APC, coliforms and *E. coli*) of broiler drumsticks (n = 5) during refrigerated storage, Experiment 3. Within days of storage, an asterisk indicates a significant difference between treatments (Jindal et al., 1995).

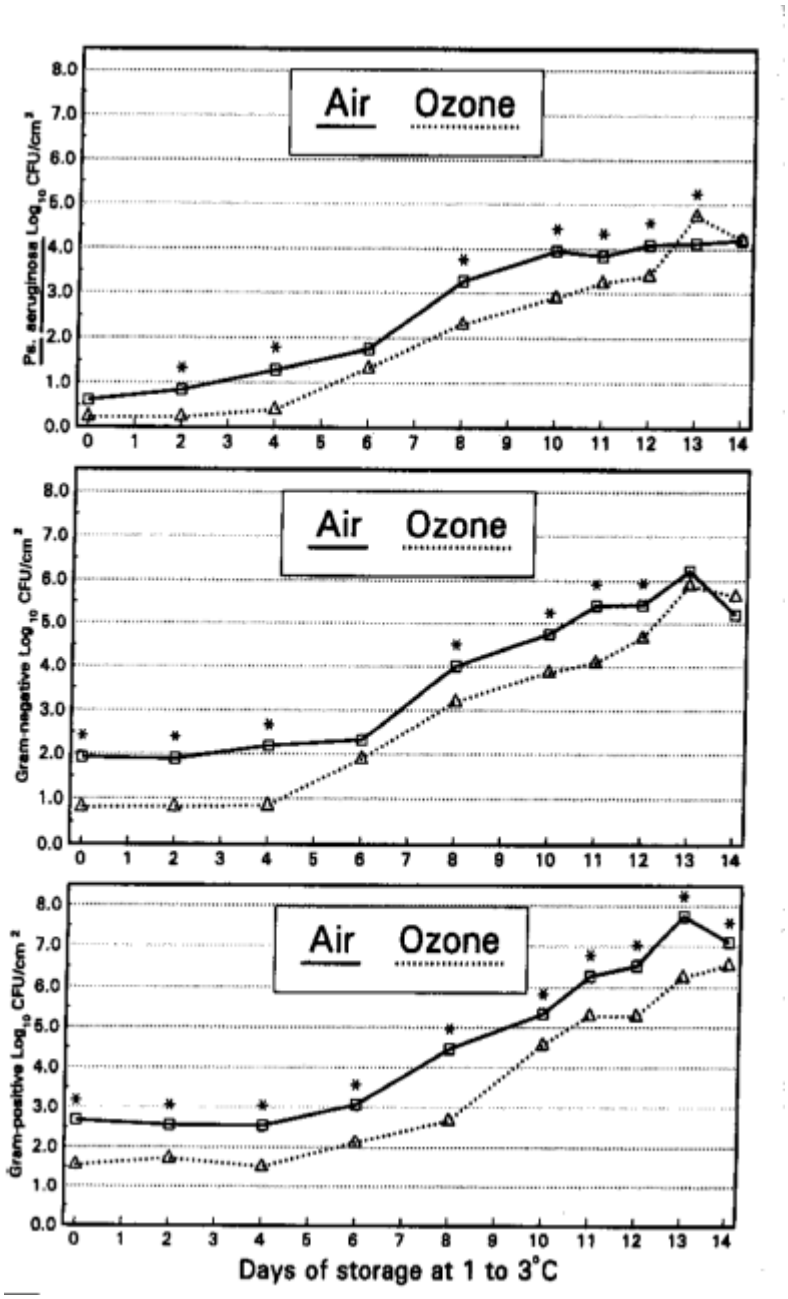


Figure 2.1.12. Effects of ozonation during immersion chilling on the microbiological quality (*Pseudomonas aeruginosa*, Gram-negative, and Gram-positive organisms) of broiler drumsticks (n = 5) during refrigerated storage, Experiment 3. Within days of storage, an asterisk indicates a significant difference between treatments (Jindal et al., 1995).

Ozonation Effects in Poultry Chill Water: In poultry chill water, ozone significantly reduced APC by 1.35 (Experiment 1), 2.72 (Experiment 2), and 2.82 (Experiment 3) logs (Table 2.1.28). In Experiment 1, the relatively high microbial load of the drumsticks increased APC levels in the chill water in both treatment groups. Therefore, reductions in microbial levels, including APC,

coliforms, and *E. coli* in Experiment 1 were comparatively lower than those noted in Experiments 2 and 3.

Table 2.1.28. Effects of ozone on the microbial quality of poultry chill water [A] (n = 2) (Jindal et al., 1995)

Treatment	APC	Coliforms	*E. coli*	*Pseudomonas aeruginosa*	Gram-negative bacteria	Gram-positive bacteria
			$\log_{10}$ CFU/mL [B]			
EXPERIMENT 1						
Air	4.12[a]	1.82[a]	1.27[a]	ND	ND	ND
Ozone	2.77[b]	0.32[b]	0.01[b]	ND	ND	ND
PSEM	0.03	0.05	0.03			
EXPERIMENT 2						
Air	3.68[a]	2.50[a]	1.93[a]	ND	ND	ND
Ozone	0.96[b]	0.02[b]	nd[b]	ND	ND	ND
PSEM	0.06	0.04	0.02			
EXPERIMENT 3						
Air	3.92[a]	1.92[a]	1.31a	1.95[a]	3.05[a]	3.86[a]
Ozone	1.10[b]	nd[b]	nd[b]	nd[b]	nd[b]	1.07[b]
PSEM	0.06	0.04	0.08	0.07	0.13	0.10

[A] Initial ozone concentration in chill water was 0.54, 0.52, and -.44 ppm, and residual ozone after 45 min of chilling was 0.06, 0.02, and 0.01 ppm in Experiments 1, 2, and 3, respectively.

[B] Lowest detection level for aerobic plate count, coliforms, and *E. coli* = $\log_{10}$ 0.00 CFU/mL. Lowest detection level for *Pseudomonas aeruginosa*, Gram-negative, and Gram-positive bacteria = $\log_{10}$ 1.0 CFU/mL.

[ND] = Not determined.

[nd] = None detected.

[a,b] Treatment means within a column with different superscripts differ significantly (P # 0.05).

Coliforms were eliminated in Experiment 3, whereas coliform levels were reduced by 1.50 and 2.48 logs in Experiments 1 and 2, respectively. *E. coli* were eliminated in Experiments 2 and 3, whereas only $\log_{10}$ 0.01 CFU/mL of *E. coli* were detected in Experiment 1. In Experiment 3, *Pseudomonas aeruginosa*, Gram-negative, and Gram-positive bacteria in the chill water also were enumerated after 45 min of treatment with air or ozone. Ozonation eliminated *Pseudomonas aeruginosa* and Gram-negative bacteria in poultry chill water. Ozonation reduced levels of Gram-positive bacteria by 2.79 logs.

Conclusions:

- Ozone can be used during immersion chilling to improve the microbiological safety of poultry products.
- Ozone treatment can extend the shelf life of poultry products during refrigerated storage.
- Ozonation can improve the microbial quality of poultry chill water by minimizing or eliminating cross-contamination during immersion chilling.

2.1.2.12 Arkansas Agricultural Experimental Station (for BOC Gases) (1997) – Macron™ Loop Evaluation

BOC Macron™ Chiller Loop Evaluation

In the summer and early fall of 1996, the BOC Macron™ Chiller Loop was evaluated during a 12-day test period at the ConAgra broiler facility in Gainesville, Georgia. The microbiological evaluation was conducted by the University of Arkansas and Woodson-Tenent, the oxidative stability assays were conducted by Woodson-Tenent, and the mutagenicity testing was conducted at the University of Florida. Dr. Amy Waldroup from the University of Arkansas served as the chain of custody during the evaluation period. Data are provided by BOC Gases, Murray Hill. NJ.

The BOC Macron™ Chiller Loop: includes a filter capable of removing solids (plus particulates, oils, grease and foam) from recycling poultry chiller water down to 25 microns coupled with ozone addition for disinfection and oxidation of some organics. Water from a chiller bath or other process areas is passed through the Macron filter, then through the ozone contact tank. A degassing unit removes dissolved gases, then the treated water is returned to the chiller for reuse (see Figure 2.1.13).

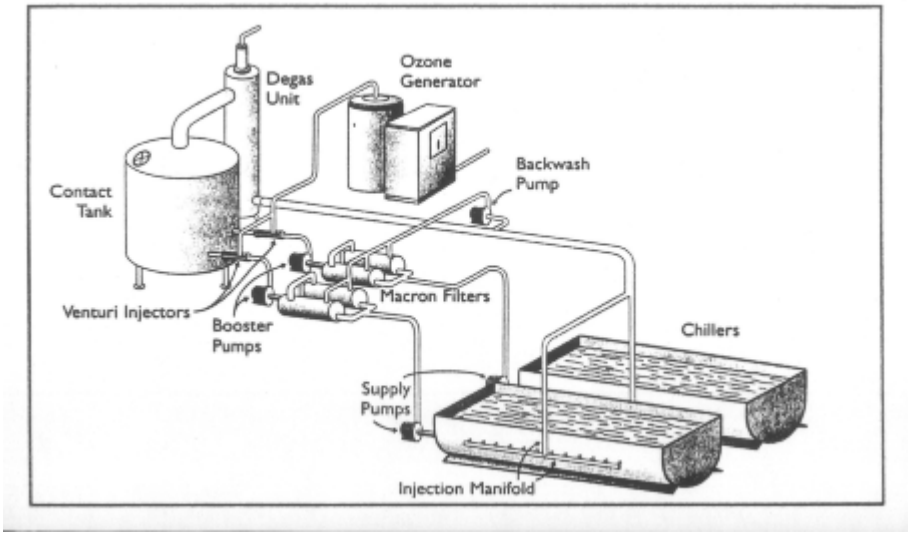


Figure 2.1.13. Schematic of the BOC Macron™ Chiller Loop (courtesy BOC Gases).

Three levels of ozone addition rates were evaluated during the test period: 16, 24, and 36 pounds of ozone/day (3.13, 4.73 and 7.09 mg/L). For each of the ozone addition levels microbial evaluations of the chiller water and chilled whole carcasses included quantification of APC (aerobic plate count), *E. coli*, coliforms; and *Campylobacter*. Incidence of *Salmonella spp*. and *Listeria monocytogenes* was determined. The oxidative stability tests included TBA values for raw and cooked broiler thighs, free fatty acid analysis, fatty acid profile of raw broiler thighs, and peroxide values of raw and cooked broiler thighs. A registered industrial hygienist also was on-site during the evaluation period to ensure that all OSHA regulations were met.

All levels of ozone reduced concentrations of APC, *E. coli* and coliforms in treated chill water by greater than 90% (Figures 2.1.14, 2.1.15 and 2.1.16 compares these microorganisms and days of storage at 4EC for the control system vs the BOC Macron™ Chiller Loop). At 24 and 36 pounds ozone/day (4.73 and 7.09 mg/L), *E. coli* and coliforms in the chill water usually were below the lower detection level (< 1 cfu/mL). At 36 pounds ozone/day (7.09 mg/L) the APC in the chill water was reduced by more than 99%. Regardless of ozone usage, levels, of *Campylobacter* in the treated chill water were reduced by 20 to 50%. Incidence of *Salmonella spp*. and *Listeria monocytogenes* were very low in both the control and treated chill water.

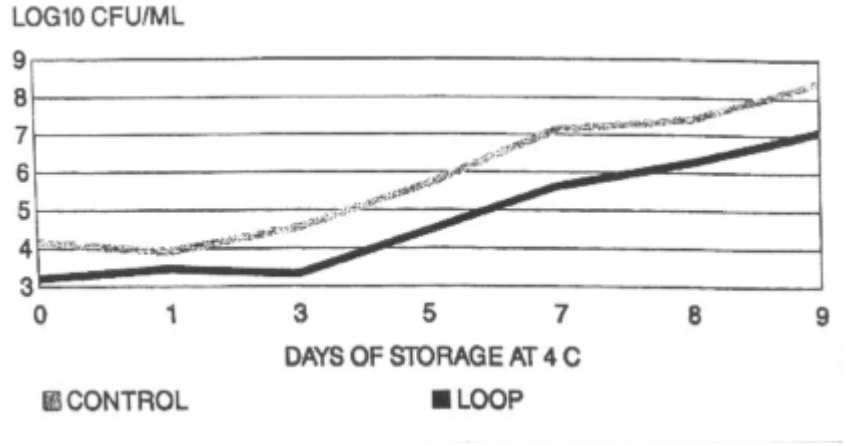


Figure 2.1.14. BOC Macron™ Loop – effects on shelflife – APC (Univ. of Arkansas, 1997)

All three levels of ozone resulted in *E. coli* levels on the carcass that were well below 100 cfu/mL; whereas counts on the control carcasses often exceeded the new FSIS acceptable range. In fact, almost 50% of the rinse samples from control carcasses exceeded 100 *E. coli*/mL. Incidence of *Sa1monella spp*. and *Listeria monocytogenes* were very low on the control and treated carcasses, indicating that this plant should be able to meet the new performance criteria for pathogens on raw broilers. Shelf life at 40EF of raw whole broilers was significantly increased (7 vs 9 days) by treatment of the chill water treatment with the BOC system (Figures 2.1.14, 2.1.15 and 2.1.16).

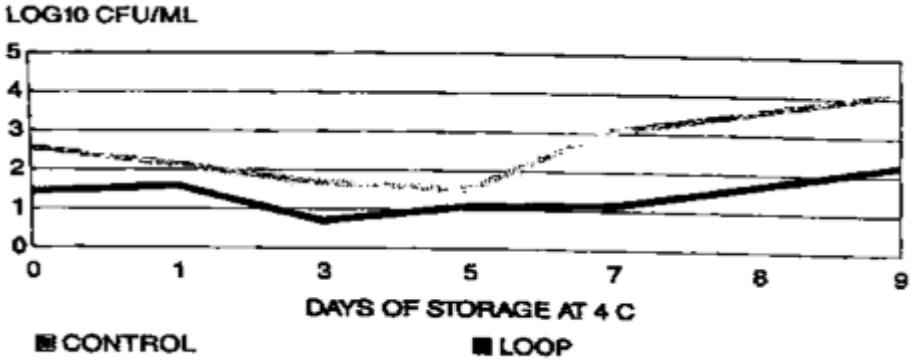


Figure 2.1.15. BOC Macron™ Loop – effects on shelflife – *E. coli* (Univ. of Arkansas, 1997)

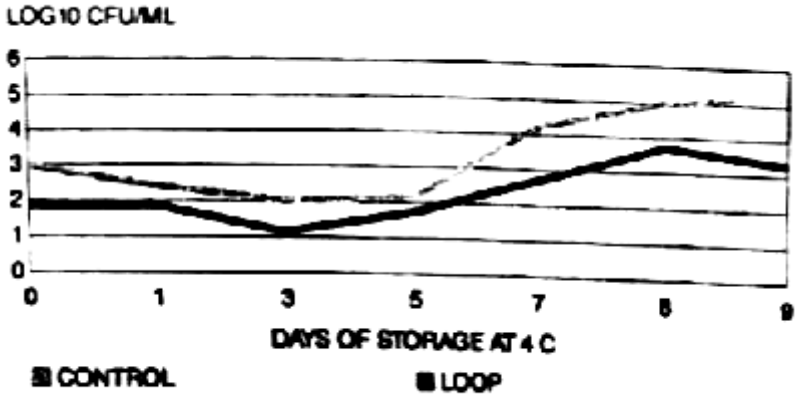


Figure 2.1.16. BOC Macron™ Loop – effects on shelflife – coliforms (Univ. of Arkansas, 1997)

All of the assays conducted to address oxidative stability of treated carcasses indicate that treatment of poultry chill water with ozone does not affect rancidity of the muscle or fat of raw or cooked poultry as measured by TBA values, peroxide values, free fatty acids, or fatty acid profile. In addition, no mutagenic compounds could be detected in broiler thighs from either the control or ozonated chill water. Numerous other reports in the literature substantiate this finding regarding lack of mutagenicity in ozone-treated poultry or other foods. During the text period the industrial hygienist did not report any problems related to use of the BOC Macron™ Chiller Loop indicating that worker safety should not be an issue.

In summary, the BOC Macron™ Chiller Loop will significantly lower the microbial levels in poultry chill water and on the post-chill carcass. In a HACCP program for raw poultry which identifies the chiller as a CCP, the system can be readily monitored, validated, and verified. The BOC Macron™ Chiller Loop should be able to maintain consistently *E. coli* levels and incidence of *Salmonella ssp*. on post-chill broilers to well within the new FSIS regulations. In addition, the process is safe, does not alter product characteristics, is environmentally and worker friendly, and cost-effective. The BOC Chiller Loop can provide the poultry industry with a very effective control measure for minimizing bacterial contamination of the ready-to-cook product without adversely affecting product quality.

2.1.2.13 FoodLabs Inc. (1998) – BOC Macron™ Loop Evaluation

During the period August 26 through September 3, 1998, FoodLabs Inc., Manhattan, KS conducted a validation of the BOC Gases Macron™ Loop Chilling System as a Post-Evisceration Critical Control Point to Reduce Pathogenic Contamination in a Commercial Poultry Slaughter Facility (Study Conducted 08126198 to 09103198, ConAgra, Gainesville, GA). The Final Report of this validation study was submitted 30 October 1998, and was made available by BOC Gases.

Executive Summary: The objective of this evaluation was to compare, in a commercial poultry processing facility, the effectiveness of an unfiltered chlorinated poultry carcass chilling system

operating under the 1/2 gallon per bird water requirement to that of a chilling system operating with the BOC Gases' Macron Loop filtration/ozonation process using chlorine dioxide in a closed system. To meet this objective, carcasses were collected immediately before chilling and after chilling over the course of eight processing shifts and were evaluated for aerobic plate counts, generic *E. coli*, and total coliform populations as well as the presence of *Salmonella* and *Campylobacter*. Additionally, water samples were collected throughout the processing shifts from the two chilling systems and analyzed for microbial populations as well as various quality parameters.

The Macron Loop system operating in a closed system (without addition of 0.5 gallon of fresh water per bird) delivered equal or better performance to that of a traditional chlorinated chiller operating under the regulatory guidelines. Total aerobic plate counts observed in the chiller water samples from the Macron Loop system were significantly lower ($p<0.05$) than those observed in the control system, with a difference of approximately 2.0 log CFU/mL.

Carcasses had significantly lower microbial populations, including total aerobic bacteria, *E. coli* and total coliform populations, as well as *Salmonella* incidence, after chilling in the Macron Loop system. Due to high variations in the microbial populations of carcasses pretreatment and the fact that the Macron Loop was not operating under optimized system conditions, it was difficult to document statistical differences in the microbial quality of carcasses chilled in the two systems. However, for all organisms evaluated, the Macron Loop delivered numerically lower average microbial populations than that of the control.

For *Salmonella*, the incidence observed before chilling was 26.6 and 18.8% for the Macron Loop and control system, respectively. After chilling, the incidence decreased to 3.1 and 7. 1 % for the Macron Loop and control systems, respectively. Importantly, a *Salmonella* incidence after chilling of greater than 0% was observed in only 3 of the 8 replications for the Macron Loop system compared to 6 of the 8 replications for the control system. Due to the sporadic nature of isolation, no definite conclusions can be drawn about the impact of the Macron Loop chilling system on the incidence of *Campylobacter*.

Evaluation of water quality data indicated that the operating parameters of the Macron Loop system were highly variable, and were likely not at optimal levels during several periods of the study. Therefore, the data collected during this study likely underestimates the true potential of the system for reducing levels of microbial populations, including the incidence of pathogens. More work is warranted to delineate the true performance advantage of the Macron Loop System.

Materials and Methods

Poultry Slaughter Facility: This study was conducted in a commercial poultry slaughter facility This facility operated two slaughter shifts, the day shift running from approximately 7 am to approximately 3:30 pm, and the night shift running from approximately 3:30 pm to approximately 12:00 am.

Chilling Systems: The slaughter facility employed the use of two chilling systems to chill freshly slaughtered poultry carcasses. One chilling system was of the type commonly used in the

poultry industry, referred to as the "control" chiller throughout this report. The second chiller, was of a similar type, however was operating on BOC Gases' Macron™ Loop filtration/ozonation system with chlorine dioxide as the oxidant. The approximate typical operating temperatures of these two systems are 38-50EF for the Macron™ Loop system and 33-43EF for the control system.

BOC Gases' staff of engineers in conjunction with ConAgra plant personnel configured the operating parameters of the Macron™ Loop chiller for the duration of the commercial trial. The BOC Macron™ Loop system operated with the addition of only enough filtered, recirculated water to maintain optimal hydraulic conditions while the unfiltered chiller operated under the regulatory requirement for the addition of 0.5 gallon of fresh water per bird processed through the chiller. Temperature of the chillers was monitored according to established plant procedures using temperature chart recorders. Copies of these charts were obtained for most of the shifts evaluated. It was noted that the Macron Loop chiller typically was 6-8EF warmer than the control chiller (Macron™ Loop chiller approximately 48EF, control chiller approximately 40EF), with a temperature differential of as much as 10EF in the later replications evaluated.

Experimental Design: The protocol used in this evaluation was developed through analysis of data obtained in preliminary commercial trials in which it was demonstrated that carcasses analyzed following chilling in the Macron™ Loop System had lower microbiological populations than carcasses analyzed prior to chilling, particularly with respect to *Salmonella* and indicators of fecal contamination. Upon review by FoodLabs' qualified statistician, it was determined that a scientifically sound protocol would require sampling over at least several shifts (eight recommended) and at equal intervals over a particular shift to account for, as best as possible, variations in poultry carcass lots, environmental conditions, water conditions, chiller conditions, etc. A diagram representing the experimental design is shown in Figure 2.1.17. Additionally, the sample collection dates and shifts associated with each sampling date are shown in Table 2.1.29.

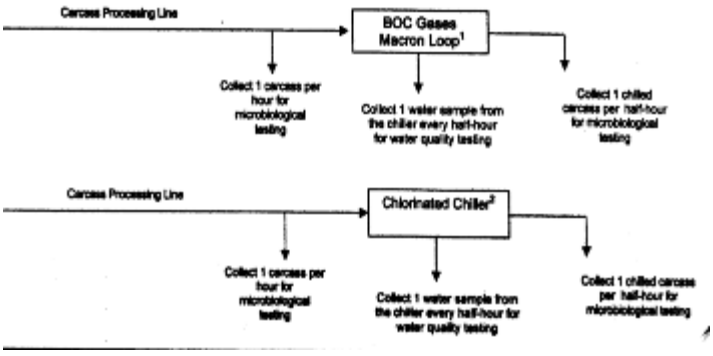


Figure 2.1.17. Experimental design. [1] The BOC Macron™ Loop Chilling unit did not operate under the 0.5 gal/bird requirement, but was supplied sufficient fresh water to maintain proper hydraulic balance. [2] The standard, unfiltered chlorinated chiller operated in accordance with the 0.5 gal/bird requirement. This protocol was repeated for eight, 8-h shifts to account for variability in carcass lots, environmental conditions, chiller conditions, etc. Carcasses were evaluated for APC, *E. coli*, coliforms, *Salmonella* and *Campylobacter*. Water samples were evaluated for generic *E. coli*, total coliforms and total aerobic plate counts, as well as pH, biochemical oxygen demand, and residual free chlorine (FoodLabs, 1998).

Although the number of shifts (replications) during which samples were collected in the commercial facility totaled 10, for two of these replications (5 and 5-b), the overnight courier failed to deliver the samples to the analytical laboratory within an acceptable time frame. Therefore, analyses were conducted and data subsequently was available for only 8 replications (no data available from replications 5 and 5-b). Analysis of replications is consistent with the original experimental design.

On each sampling day (shift), eight carcasses (total) were collected just before chilling from the two processing lines feeding into each chiller unit. These samples were scheduled to be collected at a rate of approximately one per hour for each chilling system; however the actual collection rates varied somewhat according to the conditions encountered during each slaughter shift.

Immediately after chilling, 16 carcasses (total) were collected per shift from each chiller unit. These samples originally were scheduled to be collected at a rate of one per half hour (twice as often as the samples collected before chilling). However, upon initiation of the experiment, it was suggested by BOC scientists that prior to entering the chillers, the birds to be collected after chilling should be "tagged" so as to provide a more accurate means of correlating results from samples collected before and after chilling. Unfortunately, logistics within the plant made this method unacceptable since carcasses from each chiller were intermingled shortly after they exited the chilling systems, resulting in cross contamination.

Table 2.1.29. Sample collection dates, shifts and associated times (FoodLabs, 1998)

Replication No. - Date	Shift (Day or Night)	Time Collection Initiated	Time Collection Completed
1 - 08/25/98	Day	8:50 am	3:00 pm
2 - 08/26/98	Day	9:00 am	3:40 pm
3 - 08/27/98	Day	11:20 am	3:30 pm
4 - 08/31/98	Night	4:00 pm	10:00 pm
5 - 09/01/98	Night	4:00 pm	10:30 pm
6 - 09/02/98	Night	3:15 pm	9:40 pm
7 - 09/02/98	Night	3:00 pm	9:50 pm
8 - 09/04/98	Day	9:15 am	2:00 pm
5b - 09/09/98	Night	3:15 pm	9:55 pm
9 - 09/09/98	Day	9:30 pm	2:10 pm

Therefore, for replications 1 through 4, at the time carcasses were collected before chilling, “marker carcasses" were tagged on the processing lines prior to chilling. These "marker carcasses" were used as an indication of the residence time of the carcasses in the chillers. When the "marker carcasses" were observed exiting the chillers, post-chilling samples (non-tagged) were collected. For replications 6 through 9, the use of "marker carcasses" was discontinued as their appearance at the rehanging area was determined to be sporadic. For these replications, samples were collected at designated time intervals.

In addition to carcass samples, samples of water from each chilling system were collected at a rate of one per half hour to provide a comparison of the water conditions within each chilling system. These samples were analyzed for microbial populations as well as parameters such as pH, free chlorine level, and Biochemical Oxygen Demand (BOD). For all replications, these samples were collected at a rate of approximately one per half hour.

Carcass Sampling Procedures: For collection of poultry carcasses, sample collection personnel either donned sterile gloves or inverted the sample collection bag for use as a "glove". Each carcass was placed into a sterile stomacher bag (3500 capacity) and 400 mL of 0.1% peptone diluent was poured over the carcass. After rinsing for 2 minutes, the carcass was removed from the stomacher bag using aseptic techniques and the rinse solution was transferred to a sterile bottle. One corner of the bag was sanitized using an alcohol wipe and sanitized scissors were used to trim the corner. The open corner then was used as a pour spout for transferring the liquid from the bag into a sterile plastic bottle for shipment to the analytical laboratory in Manhattan, KS.

Water Sampling Procedures: Water samples were collected from each of the chiller units during each shift to assess microbiological and quality parameters of the chiller water. At each sampling time, three water samples were collected from each chiller. One sample was collected in a sterile whirlpak bag containing a thiosulfate buffer tablet to neutralize chlorine present in the water. This sample was analyzed for total aerobic plate counts, generic *E coli*, and total coliform populations. A second water sample was collected in a sterile, empty whirlpak bag for analysis

of free chlorine and water pH. Finally, a third sample was collected in a suitable container (either rigid plastic bottle, glass bottle, or large whirlpak bag) for evaluation of the Biochemical Oxygen Demand (BOD; analyses conducted by Woodson-Tenant of Gainesville, GA). All water samples were placed immediately in refrigerated storage prior to shipment to the laboratories in appropriate containers. Water samples contained in whirlpak bags were double bagged to ensure protection against leakage during transport. For replication 9, water samples were collected for pH and chlorine determination only (no microbial or BOD analyses).

Sample Shipment to Analytical Laboratory: At the end of each sample collection shift, all carcass rinse samples and chiller water samples were packed in styrofoam coolers with gel refrigerant packs and/or ziplock bags containing wet ice. Samples were packed tightly with the refrigerant packs as well as insulating layers of plastic to reduce warming during transport. All stryofoam coolers were placed inside cardboard boxes for shipment. Shipping containers were transported by a professional overnight courier (Federal Express). Samples collected from "day" shifts were packed and transported to the courier pickup location by approximately 5-6 pm on the day of collection. These samples were shipped to arrive at the analytical laboratory by 11 am the day following collection (approximately 24 h post-sampling at the time of arrival at the analytical laboratory). For samples collected on the "night' shift, shipping containers were packed and transported to the courier pick up location by 5-6 pm of the day following collection. These samples were shipped to arrive at the analytical laboratory by 11 am on the second day following collection (approximately 36 h post-sampling at the time of arrival at the analytical laboratory). Upon receipt at the analytical laboratory, sample temperatures were evaluated to ensure the absence of temperature abuse during shipping. Upon arrival at the analytical laboratory, sample temperatures ranged from 33 to 38EF.

Microbiological Procedures - Carcass Rinse Samples: Upon receipt at the analytical laboratory, serial dilutions of the carcass rinse samples were prepared in 0.1% peptone diluent and appropriate dilutions were plated in duplicate on tryptic soy agar plates (TSA) using the spiral plate method or on Aerobic Plate Count Petrifilm plates for enumeration of total aerobic plate counts. Appropriate dilutions also were plated in duplicate on *E. coli* count Petrifilm plates for enumeration of both *E. coli* and total coliform populations. All TSA and Petrifilm plates were incubated at 35EC for 48 h. Following incubation, plates were enumerated and the colony forming units (CFU) per mL of rinse were determined for each population.

Additionally, the carcass rinse samples were analyzed for the presence of *Salmonella* spp. and *Campylobacter* spp. using qualitative procedures (VIDAS Assay, bioMerieux VITEK). For determination of *Salmonella* spp., from the original 400 mL rinse sample, 50 mL of rinse was transferred to 50 mL of double-strength Buffered Peptone Water (BPW). These samples were mixed well and incubated at 35EC for 24 h. Following incubation, 1.0 mL aliquots of the pre-enrichment were transferred to 10 mL tubes of Selenite cystine (SC) and tetrathionate (TT) broth. The SC and TT tubes were incubated at 42EC for 18 h. One mL of both of these selective enrichments then was transferred individually to 10 mL tubes of M broth, which were incubated for 24 h at 42EC. One mL from each of the M broth tubes was transferred to a sterile test tube and boiled for 15 minutes at 100EC. This boiled sample then was analyzed for the presence of *Salmonella* antigens using the VIDAS assay (bioMerieux, Hazelwood, MO). Results were reported as either "POS" or "NEG".

For determination of the presence of *Campylobacter*, 40 mL of the original 400 mL rinse sample was transferred to a sterile disposable centrifuge tube. The samples were centrifuged at 1400 rpm (16,000 x g) for 15 min. Following centrifugation, the supernatant was discarded and the pellet was re-suspended in 2.0 mL of 0.1% peptone diluent by vortexing. Samples were allowed to stand for approximately 15 minutes and this suspension was transferred to 40 mL Bolton's broth and incubated at 37EC for 6 h. Samples then were transferred to a 42EC incubator for 42 h. Following incubation, a 2.0 mL aliquot was transferred to a sterile test tube and boiled for 15 minutes at 100EC. This boiled sample then was analyzed for the presence of *Campylobacter* antigens using the VIDAS assay (bioMedeux). Results were reported as "POS" or"NEG".

Microbiological Procedures - Water Samples: Water samples for microbiological analysis were collected in sterile whirlpak bags containing thiosulfate buffer to neutralize chlorine, and thereby maintain as much as possible the microbial population present in the sample at the time of collection. Upon receipt at the analytical laboratory, water samples were serially diluted as appropriate in 0.1% peptone diluent. Appropriate dilutions then were plated on TSA agar plates using both spread plate (deposited 0.33 mL of sample onto each of 3 plates; total of 1.0 mL plated) and spiral plate techniques for enumeration of total aerobic bacterial populations. All platings were performed in duplicate. The TSA plates were incubated at 35EC for 48 h. Following incubation, plates were enumerated and the CFU/mL of water was calculated.

Water samples also were evaluated using a 3-tube Most Probable Number (MPN) technique to estimate *E. coli* and total coliform populations. Using this technique, aliquots of the water sample were transferred to 10 mL tubes of lauryl sulfate tryptose (LST) broth containing durham tubes (1.0 mL transferred into each of 3 tubes, 0.1 mL transferred into each of 3 tubes, and 0.01 mL transferred into each of 3 tubes; total of 9 tubes per water sample). These LST broth tubes were incubated at 35EC for 48 h. Following incubation, tubes were observed for positive (yellow media, gas in durum tube) or negative (no media color change, no gas in durum tube) reactions, with positive reactions indicating presumptive positive coliforms. For tubes with positive reactions, 1.0 mL of LST broth from the positive tube was transferred to 10 mL of brilliant green bile (BGB) broth. These tubes were incubated for 48 h at 35EC. Following incubation, 1.0 mL of BGB broth from positive tubes (indicating confirmed coliforms) was transferred to 10 mL of EC broth for confirmation of *E. coli*. These tubes were incubated for 48 h at 45.5EC. Positive and negative reactions were recorded, indicating confirmed *E. coli* populations. The MPN per mL of each population was calculated using values obtained from 3-tube MPN tables.

Evaluation of Water Quality Parameters: Water quality parameters evaluated in this commercial trial included pH, free chlorine, and Biochemical Oxygen Demand (BOD). The BOD samples were analyzed by a contract laboratory (Woodson-Tenent Laboratories, Gainesville, GA) in accordance with *Standard Methods For the Examination of Water and Wastewater, 18 th Edition*, 1992, APHA or EPA 600/4-79-020, Revised 1987.

The water pH was evaluated using a standardized pH meter. The probe of the meter was submerged in the water sample and the reading was recorded upon stabilization. The probe was rinsed thoroughly with distilled water and blotted dry with Kimwipes between each sample.

Free chlorine levels were evaluated using a HACH Pocket Colorimeter - Chlorine. HACH Permachern DPD Free Chlorine Reagent Powder Pillows were employed. A 10 mL aliquot of each water sample was placed in the special reagent vial and one reagent pillow was added and mixed well. The sample vial then was placed in the colorimeter and the reading was recorded (all samples evaluated with 20 seconds of reagent addition). For samples with a free chlorine value of greater than 2.2 ppm, a 1:1 dilution of the water sample was prepared using distilled water and the procedure was repeated. Results were recorded as ppm free chlorine.

RESULTS AND DISCUSSION

Water Samples: The water quality parameters measured in this evaluation included pH, free chlorine, and BOD. The mean values of these parameters for the two chillers differed for free chlorine and BOD, however no differences were observed between the mean pH values for the two chillers. The control chiller had a mean pH value of 6.6, whereas the Macron Loop chiller had a mean pH value of 6.5. Mean free chlorine levels and mean BOD levels were significantly higher (p#0.05) in the Macron™ Loop system.

In general, the pH, free chlorine, and BOD measurements all tended to indicate that the Macron™ Loop system's operating parameters were highly variable during the course of the evaluation. These data suggest that the Macron™ Loop system was not operating under optimal conditions at several points during the experiment. Therefore, it is likely that the resulting data collected during this evaluation do not accurately reflect the true potential of the system for reducing microbial loads on poultry carcasses, especially pathogenic bacterial loads.

The microbial populations observed in the water samples collected from the two chilling systems are shown in Tables 2.1.30 and 2.1.31. From Table 2.1.30, water samples collected from the Macron Loop chilling system had significantly lower (p#0.05) aerobic plate counts than did the water samples from the control system. A difference of approximately 2.0 log CFU/mL was observed.

Table 2.1.30. Mean (standard deviation) aerobic plate counts of water samples collected from the Macron Loop chilling system and the control chilling system (FoodLabs, 1998).

Replication Number	Aerobic Plate Counts			
	Log CFU/mL		CFU/mL	
	Control	Macron Loop	Control	Macron Loop
1	3.4 (0.4)	1.7 (1.0)	2,512	50
2	3.4 (0.4)	1.1 (1.0)	2,512	13
3	2.7 (0.5)	0.2 (0.5)	501	2
4	2.8 (0.3)	1.0 (0.6)	631	10
6	2.2 (0.7)	-0.0 (0.4)	158	1
7	1.7 (0.9)	0.1 (0.6)	50	1
8	2.3 (0.7)	0.5 (0.6)	200	3
9	NA [1]	NA	NA	NA
Average	2.6 [a]	0.6 [b]		

[1] NA = not analyzed; [a-b] Means with different superscripts are different (p#0.05).

For the enteric indicator organisms (Table 2.1.31) both chilling systems were found to have very low levels of generic *E. coli* and total coliforms. Mean generic *E coli* populations were <1.0 MPN/mL in all replications for both chiller types. No *E coli* populations were detected in water samples collected in five of the seven replications for the Macron Loop system compared to one of the seven replications for the control chilling system. Additionally, water samples from the Macron Loop system had mean *E. coli* populations #0.3 MPN/mL for the two replications in which this population was detected.

No statistical differences occurred in the samples from the two chilling systems for total coliform populations. Numerically, the mean total coliform population in the water samples from the Macron Loop system was lower than that in the control chiller. Because these populations were very low in both chiller systems, it is very difficult to determine the practical significance of these statistically non-significant differences.

Table 2.1.31. Mean (standard deviation) generic *E. coli* and total coliform populations (MPN/mL) in water samples collected from the Macron™ Loop chilling system and the control chilling system (FoodLabs, 1998).

Replication Number	Generic *E. coli* (MPN/mL)		Total Coliforms (MPN/mL)	
	Control	Macron Loop	Control	Macron Loop
1	0.9 (1.3)	0.3 (0.6)	5.5 (5.6)	1.1 (1.3)
2	0.1 (0.2)	0.0 (0.0)	6.7 (7.3)	1.0 (2.5)
3	0.2 (0.6)	0.0 (0.0)	1.2 (0.9)	0.0 (0.0)
4	0.2 (0.3)	0.02 (0.1)	0.5 (0.6)	0.02 (0.1)
6	0.04 (0.1)	0.0 (0.0)	0.1 (0.3)	0.0 (0.0)
7	0.0 (0.0)	0.0 (0.0)	0.2 (0.6)	0.0 (0.0)
8	0.4 (1.1)	0.0 (0.0)	0.9 (1.5)	0.0 (0.0)
9	NA [1]	NA	NA	NA
Average	0.3 [a]	0.04 [a]	1.9 [a]	0.3 [a]

[1] NA = Not analyzed.

[a] Means within population type with common superscripts and not different (p#0.05).

Carcass Rinse Samples - Quantitative Results: Tables 2.1.32 through 2.1.34 present the quantitative microbiological results from carcass rinse samples for populations of aerobic bacteria, generic *E. coli*, and total coliforms. Mean observations from all replications for both chilling systems are presented, as well as the overall means for all replications.

Before chilling, the aerobic plate counts for both chillers were 4.1 log CFU/mL (Table 2.1.32). After chilling, the APC levels were reduced (p#0.05) for both chillers by approximately 1.0 log CFU/mL. The APC populations after chilling were not different (p>0.05) for the two chilling systems (Macron™ Loop 2.9 log CFU/mL, control chiller 3.1 log CFU/mL).

A similar trend was observed for the generic *E. coli* populations (Table 2.1.33). Before chilling, the two systems had equivalent mean *E. coli* populations (2.4 log CFU/mL). For both chilling systems, the chilling process significantly (p#0.05) reduced the mean population by approximately 1.5 log CFU/mL. After chilling, the mean populations for the two chilling systems were equivalent (p>0.05; 1.0 CFU/mL for the control, 0.8 CFU/mL for the Macron Loop).

Table 2.1.32. Mean (standard deviation) total aerobic plate counts (log CFU/mL) for poultry carcasses evaluated before chilling (N=8 for each replication) and after chilling (N=16 for each replication) (FoodLabs, 1998)

Replication Number	Before Chilling		After Chilling	
	Traditional	Macron Loop	Traditional	Macron Loop
1	3.7 (0.2)	3.7 (0.5)	3.1 (0.4)	2.9 (0.2)
2	4.6 (0.8)	4.5 (0.8)	3.0 (0.2)	2.7 (0.4)
3	4.8 (0.7)	4.9 (0.9)	3.0 (0.5)	2.9 (1.0)
4	3.9 (0.5)	3.7 (0.3)	2.6 (0.5)	2.8 (0.5)
6	4.2 (0.7)	4.1 (0.5)	3.2 (0.4)	3.0 (0.3)
7	3.7 (0.5)	4.1 (0.4)	3.1 (0.3)	2.8 (0.5)
8	4.0 (0.5)	4.2 (0.4)	2.9 (0.3)	3.2 (0.6)
9	3.9 (0.3)	3.9 (0.4)	4.0 (0.7)	3.5 (0.4)
All Reps	4.1 [a]	4.1 [a]	34.1 [b]	2.9 [b]
a-b Means with different superscripts are statistically different (p#0.05).				

Carcass rinse samples from the two chilling systems had equivalent total coliform populations before chilling (approximately 2.8 log CFU/mL), as shown in Table 2.1.34. For both systems, the chilling process significantly reduced these populations by 1.0 to 1.3 log CFU/mL. For this population, the samples collected from the Macron™ Loop had statistically lower levels (p#0.05) after chilling than the samples collected from the control chiller. Although, for all bacterial populations evaluated quantitatively, the Macron™ Loop system had numerically lower populations after chilling than the control, the total coliform population was the only instance in which a statistical difference was detected among the microbial populations for the two chilling systems after chilling.

The distribution of the percent of samples within various population ranges before and after chilling for aerobic plate counts, generic *E. coli*, and total coliforms are shown in Figures 2.1.18 through 2.1.23. For all three populations, it is obvious that the chilling process, regardless of type of chiller, resulted in decreased bacterial loads on poultry carcasses.

For aerobic plate counts, before chilling (Figure 2.1.18) the distributions for both chilling systems were quite similar, with approximately 75 to 80% of the samples for both chilling systems having populations less than 4.5 log CFU/mL and no samples having populations less than 3.0 log CFU/mL. After chilling (Figure 2.1.19), the distributions for both chiller types remained similar, however approximately 60% of the samples from the Macron™ Loop system had populations less than 3.0 log CFU/mL, whereas only approximately 40% of the samples from the control chiller had populations less than 3.0 log CFU/mL.

Table 2.1.33. Mean (standard deviation) *E. coli* populations (log CFU/mL) for poultry carcasses evaluated before chilling (N=8 for each replication) and after chilling (N=16 for each replication) (FoodLabs, 1998)

Replication Number	Before Chilling		After Chilling	
	Traditional	Macron Loop	Traditional	Macron Loop
1	2.1 (0.5)	2.3 (0.7)	1.1 (0.6)	0.6 (0.6)
2	2.1 (0.4)	1.9 (0.4)	1.2 (0.3)	0.8 (0.7)
3	2.8 (0.4)	3.1 (0.4)	1.7 (0.8)	0.8 (0.6)
4	2.6 (0.7)	2.4 (0.5)	0.8 (0.7)	1.1 (0.6)
6	2.6 (0.4)	2.6 (0.5)	1.1 (0.6)	0.6 (0.7)
7	2.3 (0.5)	2.5 (0.7)	1.0 (0.5)	0.4 (0.6)
8	2.4 (0.6)	2.3 (0.7)	0.9 (0.5)	1.1 (0.9)
9	2.1 (0.5)	2.4 (0.7)	0.5 (0.8)	0.8 (0.8)
All Reps	2.4 [a]	2.4 [a]	1.0 [b]	0.8 [b]

[a-b] Means with different superscripts are statistically different (p#0.05).

Figures 2.1.20 and 2.1.21 show the distributions of *E. coli* populations for samples from the two chilling systems before and after chilling. As for the aerobic plate count populations, before chilling the distributions were quite similar, with approximately 80 to 90% of the samples for both chilling systems having populations less than 3.0 log CFU/mL and no samples having populations less than 1.0 log CFU/mL. After chilling, the overall distributions for both chillers were shifted significantly, as demonstrated by the change in mean populations (Table 2.1.33). It appears that the distribution for the Macron™ Loop system may have been shifted more dramatically than that of the control chiller, based on the fact that approximately 65% of the samples from the Macron™ Loop system had populations <1.0 log CFU/mL after chilling, whereas only approximately 45% of the samples from the control chiller had populations <1.0 log CFU/mL.

A similar trend was observed for the distributions of total coliform populations (Figures 2.1.22 and 2.1.23). In the case of this population, statistically significant differences in the mean populations after chilling for the two chilling systems were observed (Table 2.1.34). This difference also is evident in the distribution plot, with approximately 55% of the samples from the Macron™ Loop having populations <1.5 log CFU/mL after chilling whereas only approximately 30% of the samples from the control chiller had populations <1.5 log CFU/mL.

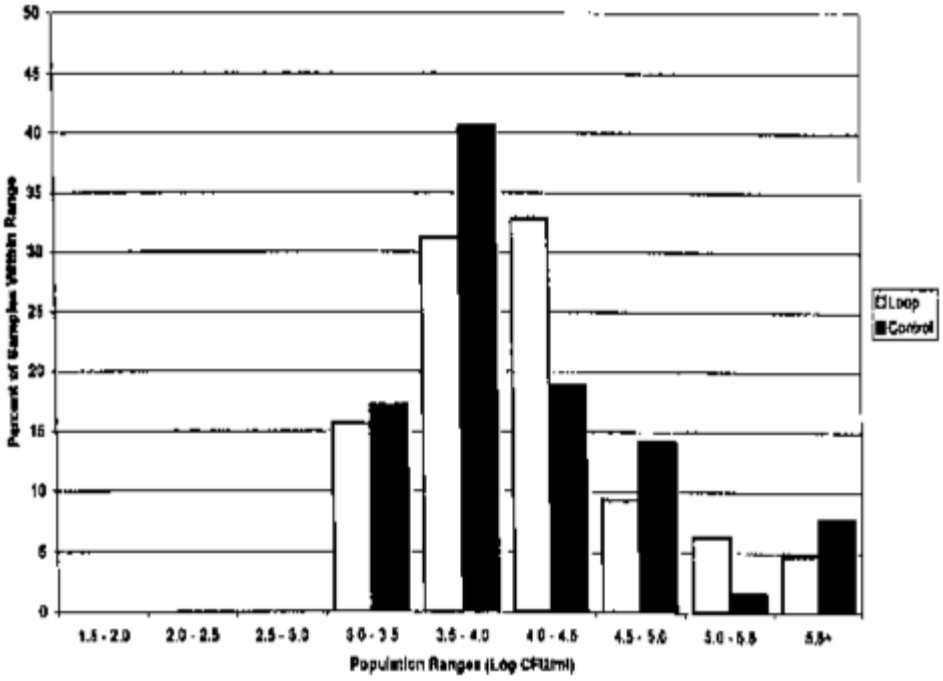


Figure 2.1.18. Distribution of aerobic plate counts on poultry carcasses before chilling (FoodLabs, 1998).

Carcass Rinse Samples - Qualitative Results: The incidence of *Salmonella* and *Campylobacter* observed for the two chilling systems before and after the chilling process are shown in Tables 2.1.35 and 2.1.36. It should be noted that the occurrence of pathogenic organisms on poultry carcasses before chilling is highly flock-dependent. Therefore, the observed incidence of these organisms is highly variable, which results in the statistical analyses being problematic to interpret. The practical significance of evaluating the statistical results for pathogen data is questionable, and therefore in addition to the statistical data, the numerical data also will be highlighted.

Statistically, no differences ($p>0.05$) were observed in the incidence of *Salmonella* before chilling for the two chillers (Table 2.1.35). The chilling process resulted in a statistically lower ($p\#0.05$) mean incidence of *Salmonella* for both chillers, with the percent of positive samples being reduced from 26.6% before chilling to 3.1% after chilling for the Macron™ Loop system (difference of 23.5%) and from 18.8% before chilling to 7.1% after chilling for the control system (difference of 11.7%). After chilling, no statistical differences were observed in the percent positive samples for the two chilling systems, even though the Macron Loop system resulted in a reduction in the incidence of *Salmonella* which was more than twice that of the control system. Importantly, in two of the three replications in which *Salmonella* was observed post-chilling for the Macron™ Loop system, operational conditions were reported by BOC Gases to be outside target specifications. Specifically, for Replication 1, the ozone treatment level was reportedly as much as four times lower than that of the remaining replications. For Replication 6, the inflow of chlorine dioxide to the system was reportedly lower than "day-shift" replications.

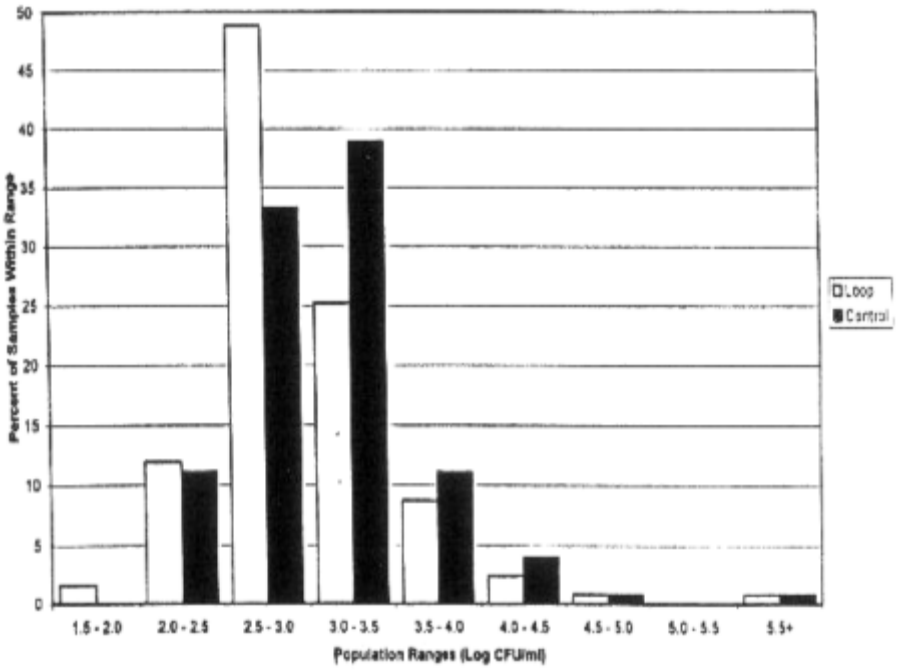


Figure 2.1.19. Distribution of aerobic plate counts on poultry carcasses after chilling (FoodLabs, 1998).

In one individual replication, the samples collected from the Macron™ Loop had a 75% incidence of *Salmonella*-positive samples before chilling and a 0% incidence after chilling (difference of 75%). The largest observed difference in percent positive samples before and after chilling for the control system was 43.7% (50% before chilling versus 6.3% after chilling). Additionally, for the Macron™ Loop system a 0% incidence of *Salmonella*-positive samples was observed after chilling in five of the eight replications. Conversely, a 0% incidence of *Salmonella*-positive samples was observed after chilling in only two of the eight replications for the control system.

The incidence of *Campylobacter*, regardless of whether it was determined before chilling or after chilling, was highly variable and appeared to be sporadic (Table 2.1.36). It should be noted that *Campylobacter* is a fastidious organism and is difficult to isolate even under optimal conditions. This variable recovery is illustrated by the fact that regardless of chiller type, the incidence before chilling ranged from 0 to 25% and after chilling the incidence ranged from 0 to 86.7%. The practical meaningfulness of the *Campylobacter* incidence data is questionable.

Table 2.1.34. Mean (standard deviation) total coliform populations (log CFU/mL) for poultry carcasses evaluated before chilling (N=8 for each replication) and after chilling (N=16 for each replication) (FoodLabs, 1998)

Replication Number	Before Chilling		After Chilling	
	Traditional	Macron Loop	Traditional	Macron Loop
1	2.7 (0.3)	2.9 (0.8)	1.7 (0.3)	1.3 (0.4)
2	2.7 (0.4)	2.6 (0.3)	1.7 (0.3)	1.4 (0.5)
3	3.4 (0.4)	3.7 (0.4)	1.9 (0.6)	1.6 (0.4)
4	3.0 (0.5)	2.8 (0.3)	1.6 (0.5)	1.6 (0.5)
6	2.8 (0.3)	2.9 (0.4)	1.8 (0.4)	1.3 (0.5)
7	2.8 (0.4)	3.0 (0.5)	1.7 (0.3)	1.3 (0.4)
8	2.9 (0.5)	2.9 (0.4)	1.6 (0.4)	1.7 (0.7)
9	2.5 (0.4)	2.5 (0.6)	2.1 (0.8)	1.4 90.4)
All Reps	2.8 [a]	2.9 [a]	1.8 [b]	1.5 [c]
[a-c] Means with different superscripts are statistically different (p#0.05).				

The mean percent of samples positive for *Campylobacter* before chilling was 10.9% for the control system and 4.7% for the Macron™ Loop system. These values were not found to be statistically different. After chilling, the percent of positive samples for the two chilling systems, which were not statistically different, was 17.9% for the control system and 4.9% for the Macron Loop system.

The high degree of variability in the occurrence, or detection of occurrence, of both the *Salmonella* and *Campylobacter* populations suggests that in order to accurately evaluate the impact of the Macron™ Loop technology on pathogen populations, one of two strategies should be employed. The first strategy would consist of conducting an experiment similar to that reported here utilizing a large number of replications with many samples collected per replication. Disadvantages would include the expense associated with such an experiment, and the inherent variability that may be nearly impossible to account for. An alternative strategy, and perhaps the preferred strategy, would be to conduct evaluations of the technology using pilot-scale equipment and inoculated carcasses. This approach would allow an accurate validation of the performance of the technology against pathogens.

Analysis of Populations Over Time: Quantitative results for carcass rinse samples were analyzed according to shifts (day versus night shift) as well as according to the time of collection within a shift. No differences were observed in the magnitude of bacterial populations as a result of shift nor were dramatic changes observed over the course of either the day or night shifts. Regression analyses of the carcass data over time were conducted. Although the regression lines do not have large R^2 values, the lines do demonstrate grossly the trends observed over sampling time (time within shift and day versus night shift). For all data sets, the trend lines either indicate relatively no change over time, or where changes were observed, the population tended to decrease slightly with sampling time.

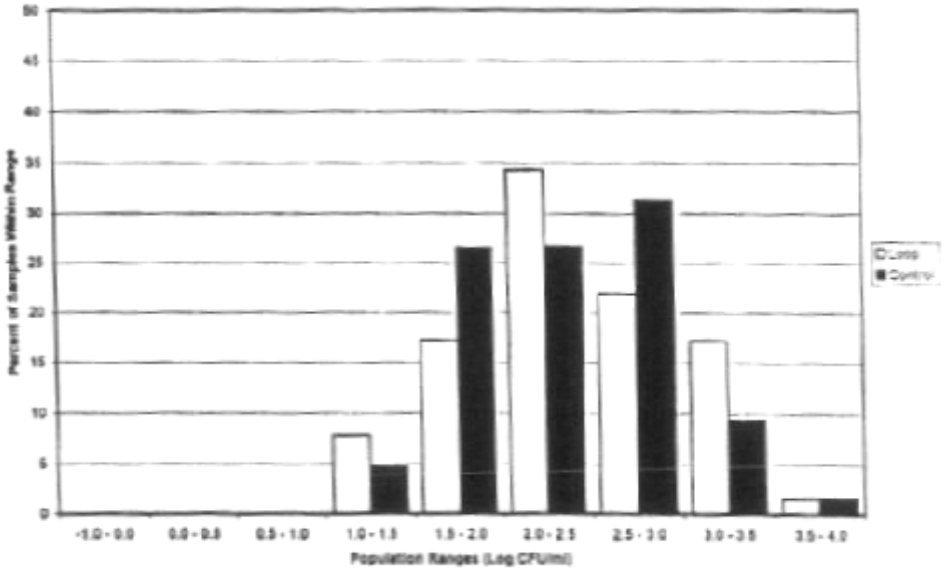


Figure 2.1.20. Distribution of *E. Coli* counts on poultry carcasses before chilling (FoodLabs, 1998).

Comparison of Generic *E. coli* Results to USDA-FSIS Pathogen Reduction Criteria: Regulations outlined in the USDA-FSIS Pathogen Reduction; HACCP Final Rule require that poultry carcasses be sampled at a rate of 1 per 22,000 and analyzed fo populations of generic *E. coli*. This regulation outlines criteria by which these results must be evaluated, with failure to meet the criteria indicating a lack of process control.

For poultry carcasses, a generic *E. coli* result of <100 CFU/mL is considered acceptable, 100 to 1000 CFU/mL is considered marginal, and >1000 CFU/mL is considered unacceptable. Evaluations are performed using the 13 most recent test results, with a "passing" status achieved by having #3 marginal results in the last 13 and no unacceptable results. A "failure" occurs when more than 3 of the last 13 test results are marginal, or when an unacceptable result is encountered.

The generic *E. coli* results of samples collected after chilling for each chiller type were evaluated using the criteria outlined in the USDA regulations to determine the number of failures for each system. It should be noted that the sampling frequency in this experimental protocol was greater than that required by the USDA regulations, and therefore the number of failures for each system indicated in this data comparison likely will be different from evaluations of the actual samples collected by the processing facility under this testing program.

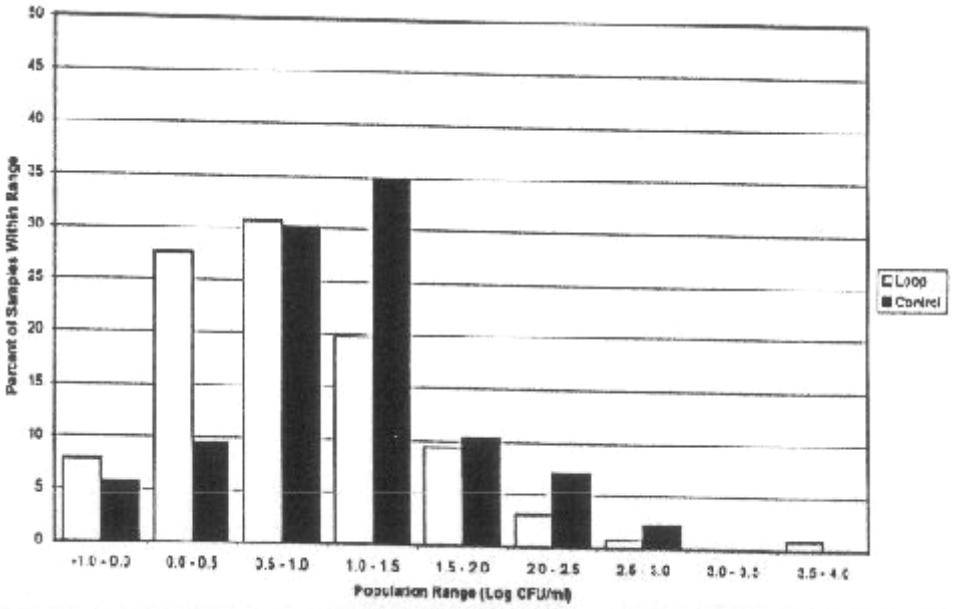


Figure 2.1.21. Distribution of *E. Coli* counts on poultry carcasses after chilling (FoodLabs, 1998).

Generic *E. coli* data from the samples collected after chilling are presented in this report as it would be for the USDA program. Process control charts have been prepared from these data for each of the chillers (Figures 2.1.24 and 2.1.25).

From these tables, no unacceptable results (>1000 CFU/mL) were encountered for either chilling system. For the control system however, 10 "failures" were observed, with all of these failures being due to excessive numbers of marginal results in the 13 most recent tests. One instance was observed in which five marginal results occurred in the most recent 13 tests. For the Macron™ Loop system, no instances of "failure" were observed, with no more than two marginal results in the most recent 13 for any given result. These results indicate that the generic *E. coli* process control data was impacted positively by the Macron™ Loop chilling system as compared to the control chilling system.

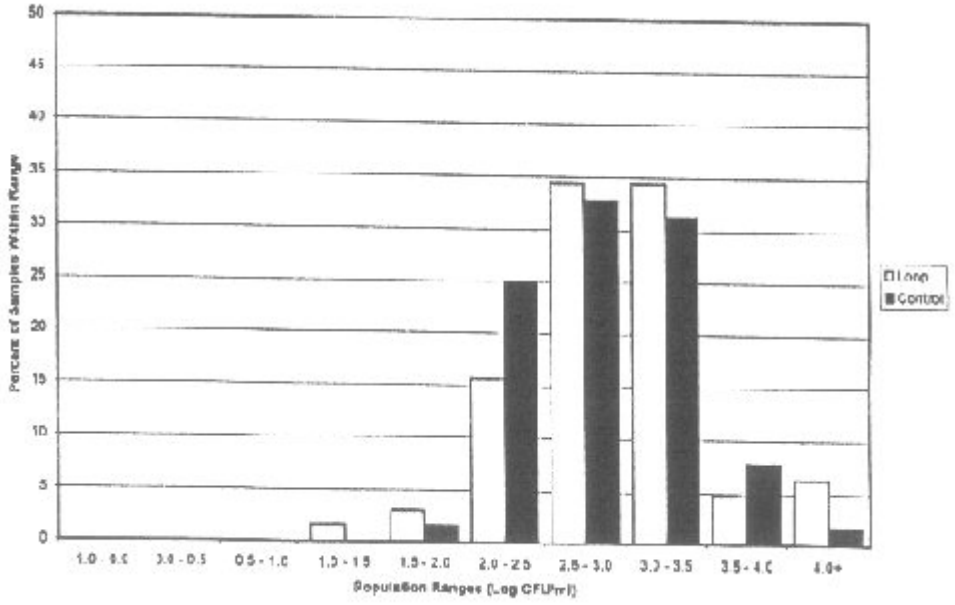


Figure 2.1.22. Distribution of coliform counts on poultry carcasses before chilling (FoodLabs, 1998).

Major Conclusions: The following major conclusions can be made from data presented in the previous sections:

1. The Macron™ Loop system operating in a closed system (without addition of 0.5 gallon of fresh water per bird) delivered equal or better performance to that of a traditional chlorinated chiller operating under the regulatory guidelines. Total aerobic plate counts observed in the water samples from the Macron™ Loop system were significantly lower (p#0.05) than those observed in the control system, with a difference of approximately 2.0 log CFU/mL.

2. Carcasses had significantly lower microbial populations, including total aerobic bacteria, *E. coli* and total coliform populations, as well as *Salmonella* incidence, after chilling in the Macron™ Loop system. Under the conditions of this evaluation, high variations in the microbial populations of carcasses pretreatment, it was difficult to document statistical differences in the microbial quality of carcasses chilled in the two systems. However, for all organisms evaluated, the Macron™ Loop delivered numerically lower average microbial populations than those of the control.

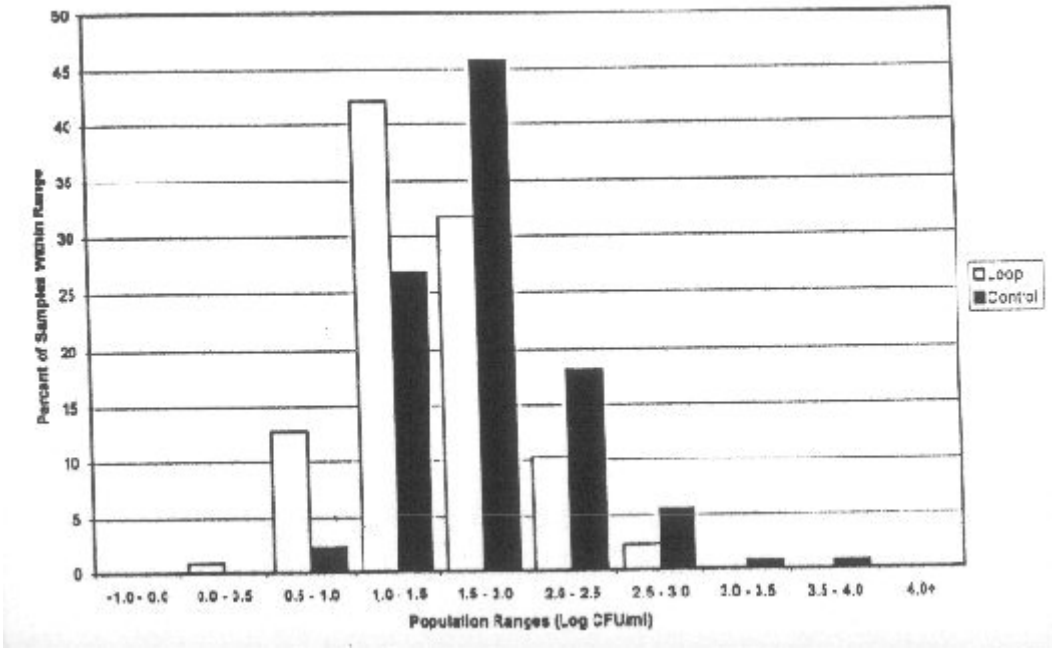


Figure 2.1.23. Distribution of coliform counts on poultry carcasses after chilling (FoodLabs, 1998).

3. For *Salmonella*, the incidence observed before chilling was 26.6 and 18.8% for the Macron™ Loop and control system, respectively. After chilling, the incidence decreased to 3.1 and 7. 1 % for the Macron™ Loop and control systems, respectively. While the Macron™ Loop delivered a greater average reduction in the incidence of *Salmonella* under the conditions of this evaluation, high variability in the incidence of this organism due to flock variations before chilling likely impacted the inability to detect statistical differences in the performance of the two chilling systems. To address the inherent problem of flock variability, it would be necessary to either sample significantly more carcasses in more replications, or preferably, perform an inoculated validation study under controlled conditions (i.e., pilot-scale evaluations). This flock variability, coupled with the fastidious nature of the organism, amplified this problem in the case of *Campylobacter*.

4. In comparing the generic *E. coli* data to criteria outlined in the USDA Mega Reg, no instances of process failure were observed for the Macron™ Loop system. Conversely, 10 instances of process failure were observed for the chlorinated control system. It should be noted that the sample collection frequency in this evaluation differed somewhat from that which would be utilized under these regulations.

Table 2.1.35. Incidence of *Salmonella* spp. (% positive) for poultry carcasses evaluated before chilling (N=8 for each replication) and after chilling (N=16 for each replication) (FoodLabs, 1998)

Replication Number	Before Chilling		After Chilling	
	Traditional	Macron Loop	Traditional	Macron Loop
1	12.5	25.0	18.8	6.3
2	12.5	12.5	6.3	0.0
3	25.0	75.0	6.3	0.0
4	12.5	25.0	6.3	0.0
6	50.0	37.5	6.3	12.5
7	12.5	0.0	0.0	0.0
8	25.0	25.0	12.5	6.3
9	0.0	12.5	0.0	0.0
All Reps	18.8% [a]	26.6% [a]	7.1% [b]	3.1% [b]

[a-b] Means with different superscripts are statistically different (p#0.05).

Table 2.1.36. Incidence of *Campylobacter* spp. (% positive) for poultry carcasses evaluated before chilling (N=8 for each replication) and after chilling (N=16 for each replication) (FoodLabs, 1998)

Replication Number	Before Chilling		After Chilling	
	Traditional	Macron Loop	Traditional	Macron Loop
1	0.0	0.0	18.8	0.0
2	25.0	12.5	6.3	0.0
3	0.0	0.0	0.0	0.0
4	12.5	12.5	31.3	6.3
6	0.0	0.0	0.0	6.3
7	25.0	0.0	0.0	6.3
8	12.5	0.0	0.0	0.0
9	12.5	12.5	86.7	20.0
All Reps	10.9% [a]	4.7% [a]	17.9% [a]	4.9% [a]

[a] Means with different superscripts are statistically different (p#0.05).

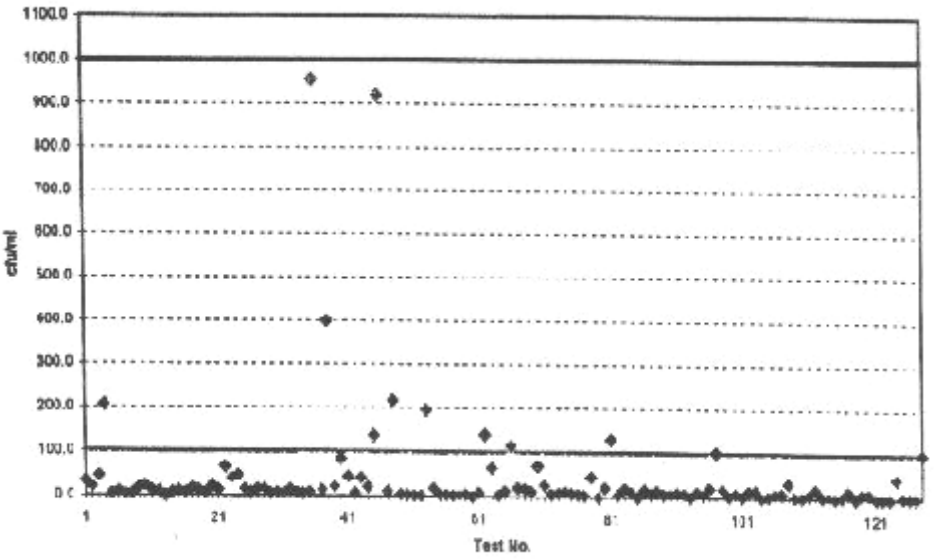


Figure 2.1.24. Process control chart for generic *E. coli* -- control chiller (Foodlabs, 1998).

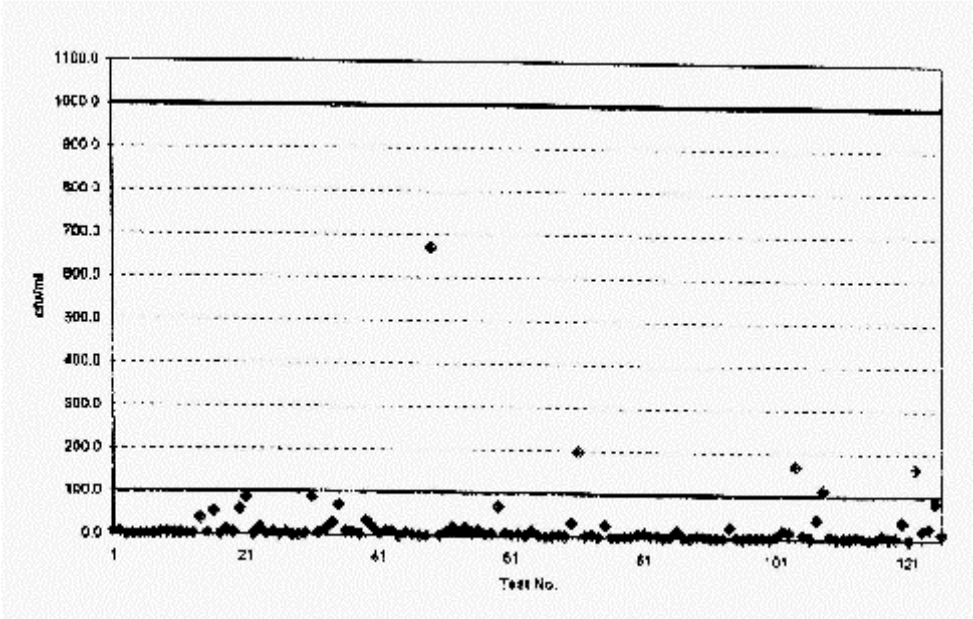


Figure 2.1.25. Process control chart for generic *E. coli* -- Macron™ Loop chiller (Foodlabs, 1998).

2.1.2.14 Diaz and Law (1999) – UV-Ozonation of Chiller Water

Abstract: UV-enhanced ozonation experiments on unscreened overflow chiller-water samples from a commercial poultry-processing plant successfully compared the oxidative and bactericidal

effects of four treatments (viz., O_2/O_3, O_2/UV, O_2/O_3/UV, and O_2 as the control). At least a 60% reduction of total microorganism levels including similar reductions in levels of coliforms and *E. coli* as well as the maintenance of light transmission at a value no less than 80% that of fresh water were obtained. This satisfies USDA-FSIS regulations that permit 90% recirculation of 0.5 gal (1.9 L) overflow per carcass required. Also an additional reduction (>0.8 Log, CFU/mL) in aerobic plate count (APC) was documented for ozone acting in concert with UV photons as compared with the sum of the effects of O3 and UV acting separately in series. Economic analysis of operational and maintenance costs for the UV-enhanced ozonation system recommended from this work estimates annual savings of ~$244,000 in a 1/4-million bird per day plant achieving water savings of 426,000 L/day (113,000 gal/day).

Experimental Design: The proposed treatment method is an advanced oxidation process (AOP) of UV-enhanced ozonation (O_3/UV) for improving the microbiological safety, turbidity, and water-use efficiency of overflow poultry chill water allowing its reconditioning for reuse. Experimentation was conducted in two phases. The first phase involved tests utilizing broiler overflow chiller water samples to understand the enhanced effect of the ozone-UV process under replicable conditions. The second phase involved determining the effectiveness of the O_3/UV process in eliminating *Salmonella* from chiller water.

Chiller Wastewater Trial: This phase of experiments treated overflow chiller water from a number of sampling days. Overflow chiller water samples were obtained from a nearby broiler processing plant during the period of March through November 1997. Preliminary sampling at different daily periods and stages of the processing cycle indicated wide variability in the water quality parameters. Peak microbial and organic levels occurred after the last carcass of the second work shift entered the pre-chiller prior to the sanitation shift (i.e., when the chillers were emptied and cleaned following 16 h of use). It is at this sampling time that the real-life efficacy and efficiency of the AOP process in treating poultry overflow chiller water was evaluated.

The experiments were performed for random treatment combinations of three ozone and UV levels, examined at contact times of 4, 8, and 16 min. The applied ozone dose levels were 0, 2.55∀0.21, and 5.11∀0.42 mg/L-min; the UV power levels were 0, 295 and 428 mW/L. A 10 L batch of overflow chiller water was transported to the laboratory and maintained at 4EC. Before and after treatment the batch was sampled to determine water quality parameters: total suspended solids (TSS), percent light transmission (%LT) at 500 nm, chemical oxygen demand (COD), *E. coli*, total coliforms (TC), and aerobic plate count (APC). *E coli*, TC, and APC were enumerated using the Petrifilm™ Plate Method.

The stock sample was used to obtain 250 mL sample volumes per reactor which comprised a single experimental sample. Three grouped reactors comprised a single experimental run or block. Nine of these blocks made up a single experimental replication. A factorial experimental design allowed testing for interactions with at least three replications comprising a cell or block. Three factors at different levels each were established prior to commencing treatment and evaluated following it: ozone dosage, UV radiation intensity, and contact time. Responses measured following treatment were the water quality parameters already mentioned.

This phase showed the effectiveness and kinetics of the system utilizing process water. It determined the ozone and UV combination that, within the least amount of time, disinfected the

chiller water to meet current USDA regulations. Also, this phase determined how changes in ozone concentrations and UV intensities affected the microbial concentration and light transmission of poultry wastewater.

***Salmonella typhimurium* Trial:** It was necessary to numerically quantify the reductions in *Salmonella* levels before and after treatment without the use of expensive antigen detection kits that screen foods and feeds for the presence of the *Salmonella* species and only provide culture confirmation (i.e., presumptive positive). An alternative method was to use a modified strain of Nalidixic Acid (NAL) resistant *Salmonella* strain 4415 (mutant of strain 3333/0) from the American Type Culture Collection (ATCC) surface/spread plated in duplicate 0.1 mL (i.e., 10^{-1} dilution) samples on Brilliant Green (BG) Sulfa Agar plates containing ~200 ppm NAL Acid as the selective medium. The purpose of the agar is to be selective for gram- microbes while the NAL selects for gram+, therefore allowing the growth of only the inoculated NAL resistant *Salmonella* in the wastewater that was treated.

The strain was inoculated from two beads, kept in a cryoprotective medium at -80EC, into a tube containing 9 mL of tryptic soya broth (TSB) containing -200 ppm of NAL and then incubated at 35EC for 24 h. From this culture 1 mL was transferred into another TSB+NAL tube and incubated at 35EC for 24 h; the *S. typhimurium* then were acclimated to cold temperatures at 4-8EC for 24 h. Before the experiment, 10 mL of this suspension was diluted 1:1000 with 10 L of previously sampled broiler overflow chiller wastewater (obtained earlier from the plant and stored at 4EC) to obtain an initial plate count of Log 5.5 CFU/mL. The inoculated wastewater was kept at 4EC to maintain constant *S. typhimurium* numbers throughout the experiment. Serial dilutions for plating were done using 9 mL of sterile, 6.8% phosphate buffer. The plates were incubated at 35EC for 24 h (no difference in counts at 48 h). The 10 L stock sample was used to obtain 250 mL sample volumes per reactor. Each 250 mL volume of pre-chiller overflow wastewater comprised a single experimental sample. Six grouped reactors comprised a single experimental run or block. Two of these blocks made up a single experimental replication. This phase utilized the optimal settings of UV and ozone dosages obtained in the previous phase (i.e., 5.11 mg/L-min and 295 mW/L, respectively). *S. typhimurium* counts were recorded as the number of colony forming units (CFUs) per plate. Counts were analyzed statistically by the GLM procedure after a log transformation; this was necessary in order to bring the distribution of the data closer to normality since microbiological data in its raw form does not produce the normal distribution needed for meaningful statistical analysis. A minimum of three replicates was needed to obtain a balanced 3x2x2 factorial experiment in blocks of size six. Comparisons between O_3 vs UV, O_3 vs O_3/UV, and UV vs. O_3/UV were performed.

This phase determined if ozone with UV is effective for reducing or eliminating levels of *Salmonella* in poultry chiller overflow water consistent with USDA (1997) recycling standards which require the reduction of at least 60% of total microorganisms including *Salmonella spp*.

RESULTS AND DISCUSSION

Chiller Wastewater Trial: A synergistic effect (i.e., > 0.8-log CFU/mL additional reduction) in APC levels was obtained for ozone acting in concert with UV photons as compared with the sum of the effects of O_3 and UV acting separately (Table 2.1.37). As expected, no reductions in levels of APC, *E. coli*, and TC resulted from only the background oxygen flow. UV dosages of

178 and 247 mW/L very effectively eliminated bacterial counts (>90% for *E. coli*, TC, and APC) for all treatment durations tested. Even though, at the dosages tested, ozone alone was not as effective as UV in reducing the microbial load, both dosages did provide >90% reduction in levels of *E. coli* and TC and >80% reduction in APC levels after 16 min of treatment. During the same treatment time with an ozone dosage of 5.11 mg/L-min, an apparent synergistic effect between O_3 and UV dosage of 295 mW/L was obtained in APC reduction (Table 2.1.38). Statistical analysis revealed significant differences ($P<0.01$) for the main effects of Ozone and Time for all the parameters tested. As was expected UV did have a significant effect ($P<0.05$) on all of the microbiological parameters (i.e., *E. coli*, TC, and APC). The important interaction of Ozone x UV had the greatest effect ($P<0.01$) on all the microbiological parameters, thus suggesting a synergistic effect between ozone and UV irradiation for the reduction of microorganism levels from poultry chiller wastewater. No significant effects ($P>0.10$) due to the different reactors used for experimentation was shown ($P>0.05$ for % LT). Profile plots of the Ozone x UV interaction revealed that no additional reductions in APC levels were achieved with a UV dosage of 428 mW/L when compared to 295 mW/L. The same figure showed that an ozone dosage of 2.55 mg/L-min had no added reductions in APC levels when compared to 0 mg/L-min.

The above results explain the reduced bactericidal effect of ozone in the presence of soluble organic materials as reported by Yang and Chen (1979). As more ozone is being utilized and self-decomposed in the presence of organic matter, less becomes available to impact the microbiology of the water. The most effective settings of ozone and UV dosages for this test phase were 5.11 mg/L-min and 295 mW/L, respectively. These dosages thus were used in the second phase for the reduction of *Salmonella typhimurium* levels.

A decrease in levels of TSS and COD (361 to 249 mg/L and 1731 to 1498 mg/L, respectively) and a small increase in %LT were documented (50.8 to 55.7) after 16 minutes of treatment with only background oxygen flow. Similar results were obtained in TSS, %LT, and COD with UV dosages of 178 and 247 mW/L for all treatment durations tested. Both ozone dosages were very effective in reducing levels of TSS (<132 mg/L) and COD (<1200 mg/L), and increasing %LT (>70%) after 16 min of treatment. Statistical analysis revealed significant differences ($P<0.01$) for the main effects of Ozone and Time for all of the chemical and biochemical parameters tested. Interestingly, UV had a significant effect on %LT ($P<0.05$) and none on TSS and COD. The interaction of Ozone x UV had no significant effect ($P>0.10$) on the same parameters.

Table 2.1.37. Effect of Different Treatment Combinations of O_3 and UV on Several Water Quality Parameters in Broiler Overflow Chiller Wastewater after 16 Minutes Treatment Duration[a] (Diaz and Law, 1999)

Treatment	TSS, mg/L	%LT	COD, mg/L	*E. coli*, Log CFU/mL	TC, Log CFU/mL	APC, Log CFU/mL
Untreated	361	50.8	1731	2.3	2.6	4.1
O_2	249	55.7	1498	2.3	2.6	4.1
O_2/O_3	122	74.5	1066	0.7	0.9	3.4
O_2/UV	248	55.2	1494	0.7	0.8	2.7
O_2/O_3/UV	123	80.4	1068	0.7	0.7	1.1
[a] Ozone dosage of 5.11 mg/L-min and UV dosage of 295 mW/L.						

Table 2.1.38. Effect of UV-Enhanced Ozonation on Several Water Quality Parameters in Broiler Overflow Chiller Wastewater as Functions of Treatment Duration [a] (Diaz and Law, 1999)

Time, min	TSS, mg/L	%LT	COD, mg/L	*E. coli*, Log CFU/mL	TC, Log CFU/mL	APC, Log CFU/mL
0	361	50.8	1731	2.3	2.6	4.1
4	194	55.2	1285	0.7	0.8	2.5
8	167	62.0	1170	0.7	0.7	1.6
16	123	80.4	1068	0.7	0.7	1.1
[a] Ozone dosage of 5.11 mg/L-min and UV dosage of 295 mW/L.						

***Salmonella typhimurium* Trial:** The antimicrobial effects of the best settings of UV and ozone dosages, obtained in the previous phase (i.e., 5.11 mg/L.min and 295 mW/L, respectively), against *Salmonella* are plotted in Figure 2.1.26 as functions of treatment duration. Reductions of >0.9 Log (i.e., 87.4%) after 8 min of ozonation were achieved using 5.11 mg/L-min; in comparison the UV intensities utilized were more effective by providing 2.6-log (>99.9%) reductions in *Salmonella* levels with 295 mW/L dose after 8 min.

At 4 to 8 min treatment times a synergistic bactericidal effect was documented between O_3 and UV acting simultaneously. This accounts for an additional reduction in *Salmonella* level (>1.l-log). As confirmed in Figure 2.1.26, no reduction in level resulted from only the background oxygen flow and magnetic stirring. Statistical analysis revealed significant differences ($P<0.01$) for all main effects as well as their interactions. Of all the interaction effects, Ozone x UV was greatest, again suggesting that a synergistic effect between ozone and UV irradiation occurred.

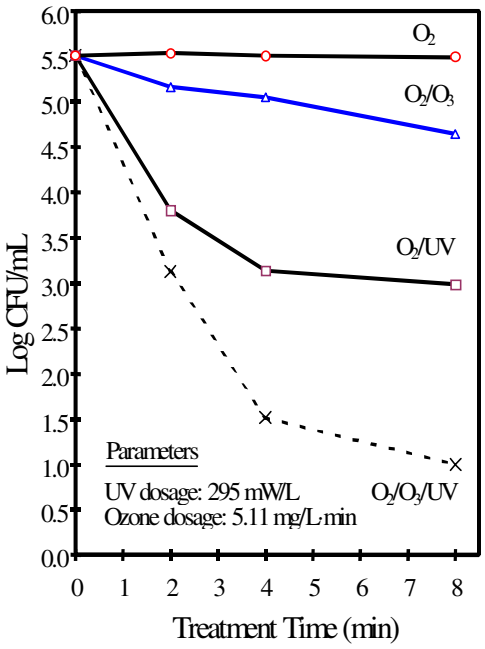


Figure 2.1.26. Bactericidal effect of three different treatments on NAL acid-resistant *Salmonella typhimurium* inoculated broiler overflow chiller wastewater as functions of treatment duration (Diaz and Law, 1999).

Conclusions:

At least a 60% reduction of total microorganisms, including similar reductions in coliforms and *E. coli* as well as the maintenance of light transmission at a value no less than 80% that of fresh water, were obtained by the four treatment processes tested. An additional reduction (>0.8 Log CFU/mL) in APC has now been documented for ozone acting in concert with UV photons as compared with the sum of the effects of O_3 and UV acting separately. Even though ozone alone, at the dosages tested, was not as effective as UV in reducing the microbial load, both ozone dosages did provide >90% reduction in levels of *E. coli* and TC and >80% reduction in APC after 16 min of treatment. Both ozone dosages were very effective in reducing levels of TSS (<132 mg/L) and COD (<1200 mg/L), and increasing %LT (>70%) after 16 min of treatment. During the same treatment time with an ozone dosage of 5.10 mg/L-min, an apparent synergistic effect between O_3 and UV dosage of 178 mW/L was obtained in APC level reduction. These results indicate a 90% closed-loop chill-water recirculation could be obtained consistent with USDA-FSIS guidelines.

The second phase effectively reduced the level of NAL resistant *Salmonella typhimurium* inoculated in poultry overflow chiller water. Reductions of >0.9 Log (i.e., 87.4%) and 2.6 Log 99.9%) after 8 min of ozonation were achieved using 5.11 mg -min ozone and 295 mW/L UV, respectively. A synergistic bactericidal effect was documented between O_3 and UV acting simultaneously at 4 to 8 min.

2.1.2.15 EPRI (1999b) – Chiller Water Ultrafiltrate

The objectives of this study were to address two broad issues -- (a) evaluation of the feasibility of reduction of water use in poultry chiller operation by membrane filtration, and (b) evaluation of the efficacy of ozonation as an alternative to chlorination in chiller operation starting with the chiller water ultrafiltrate.

Ozonation of Chiller Overflow Ultrafiltrate: Chiller water overflow was treated by a Koch HFK131 ultrafiltration membrane. About 300 liters of the permeate was collected in the contactor tank for this purpose. The COD level of the collected ultrafiltrate was about 200 to 300 mg/L. Total chlorine content in the ultrafiltrate was about 13 mg/L, which would interfere with ozone analysis. Therefore, sodium thiosulfate was used to neutralize chlorine in the ultrafiltrate before addition of ozone.

Dechlorinated ultrafiltrate was recirculated through the ozonation apparatus. Aqueous ozone concentration reached about 1.5 mg/L within the first 5 minutes of ozonation, then the rate of increase slowed down. About 120 minutes of ozonation was required for the concentration to reach 4 mg/L. Treatment of broiler carcasses with ozonated UF chiller water containing 1.5 to 2 mg/L did not show any significant microbiocidal benefit, perhaps due to the high level of COD (200-300 mg/L).

Treatment of Pre-chiller Carcasses with Ozonated Tap Water: Tap water ozonated for about ten minutes reached ozone levels in the range of 6 to 8 mg/L. A trial was conducted with tap water ozonated for ten minutes to evaluate the effectiveness of high ozone level on microbial counts in chicken carcasses. The results of this trial are summarized in Table 2.1.39. The results of this trial indicated that tap water with 6-8 mg/L ozone level was effective in reduction of microbial counts in 30 minute contact times allowed in the trial. This trial confirmed that the lower ozone level in the previous trial with chiller overflow ultrafiltrate was a primary reason for ineffectiveness of the treatment. The demand for ozone by the organic matter in the chiller overflow ultrafiltrate prevented reaching adequate ozone levels in a reasonable length of time.

Treatment of Pre-chiller Carcasses with Ozonated Chiller Overflow Ultrafiltrate with Hydrogen Peroxide: Carcasses were subjected to a thorough 30 second inside-outside rinse with ozonated ultrafiltrate prior to soaking in the contactor tank. Hydrogen peroxide was added as a secondary oxidizer, using a commercial solution of 3% hydrogen peroxide in water. Initially, 18 mL of this solution was added to 400 L volume of the contactor tank. This was followed by continuous addition of 4.5 mL/min of the same solution. These rates maintained a molar ratio of 4 between ozone and hydrogen peroxide. The results of this trial are summarized in Table 2.1.40.

Table 2.1.39. *E. coli* Analysis of Carcasses Treated with Ozonated Tap Water; Soak for 30 min (EPRI 1999b)

Carcass Number	March 23 Control	Treatment
1	80	< 10
2	180	40
3	3,100	40
4	40	30
5	> 40,000	30
Average	Ind.	35
Total	Ind.	140

Table 2.1.40. E. Coli Analysis of Carcasses Treated with Ozonated Chiller Overflow Ultrafiltrate with Hydrogen Peroxide Rinse for 30 sec and Soak for 30 min (EPRI, 1999b)

Carcass Number	April 9 Control	Treatment
1	1,600	50
2	1,000	30
3	130	70
4	50	30
5	60	< 10
Average	568	45
Total	2,840	180

The results of this trial indicated that chiller overflow ultrafiltrate with 1 to 2 mg/L ozone level was effective in reduction of microbial counts in 30 minute contact time allowed in the trial when combined with about 0.05 mg/L of 3% hydrogen peroxide as a secondary oxidizer.

Treatment of Post-chiller Carcasses with Ozonated Chiller Overflow Ultrafiltrate with Hydrogen Peroxide: Rinsing the carcasses leaving the chiller with chiller overflow ultrafiltrate is another possible approach for reduction of microbial counts using ozone. Two trials were conducted to evaluate the effectiveness this approach. The procedure followed in these trials was similar to the previous tests, except that the carcasses were not allowed to soak in the contactor tank. Instead, the carcasses were sampled immediately following a 30 second rinse. The results of these trials are summarized in Table 2.1.41. The results of this trial indicated that a 30 second rinse with chiller overflow ultrafiltrate with 1 to 2 mg/L ozone level and 0.5 mg/L hydrogen peroxide was effective in reduction of microbial counts in carcasses.

Table 2.1.41. *E. Coli* Analysis of Carcasses Treated in Ozonated Chiller Overflow Ultrafiltrate with Hydrogen Peroxide Rinse for 30 sec (EPRI, 1999b)

Carcass Number	April 9 Control	Treatment	April 15 Control	Treatment
1	10	20	40,000	30
2	< 10	20	40,000	20
3	180	130	40,000	10
4	10	20	40,000	20
5	< 10	30	40,000	50
Average	67	44	40,000	26
Total	200	220	200,000	130

2.1.3 Poultry – Microbiological Studies in Air

2.1.3.1 Whistler and Sheldon (1989b) – Hatchery Disinfectant

Abstract: Ozone was evaluated as an alternative hatchery disinfectant to replace formaldehyde. Cultures of *Staphylococcus, Streptococcus*, and *Bacillus* species previously isolated from poultry hatcheries and selected culture collections of *Escherichia coli, Pseudomonas fluorescens*, *Salmonella typhimurium*, *Proteus* species, and *Aspergillus fumigatus* were spread-plated on open petri plates and independently fumigated with ozone or formaldehyde in a prototype laboratory poultry setter. Ozone (1.52% to 1.65% by weight) resulted in reductions in bacterial levels of >4 to 7 $\log_{10}$ and fungal reductions of >4 $\log_{10}$, whereas formaldehyde achieved reductions of >7 $\log_{10}$ and >5 $\log_{10}$, respectively, after 8 min of exposure to either disinfectant. Potential mutagenic effects were observed in ozonated *E coli* colonies resulting in decreased superoxide dismutase activity and increased catalase activity when compared with nonozonated control colonies. In this study ozone reduced microorganism counts but not as much as formaldehyde. Ozone may be used as a disinfectant against selected microorganisms. although further testing under actual hatchery conditions is needed before making recommendations to the industry.

Materials and Methods:

Test Organisms: Cultures were obtained from one of three sources. *Bacillus, Streptococcus*, and *Staphylococcus* species were isolated from the sampling of the North Carolina State University (NCSU) Research Unit Hatchery; *E. coli* was from the American Type Culture Collection (ATCQ 33625; *Salmonella typhimurium* B-13, *P. fluorescens* B-14, and *Proteus* B-13 were from M. Daeschel (USDA Fermentation Lab, Dept. of Food Science, NCSU); and *A. fumigatus* was from ATCC 26933. The microorganisms tested in this study either have been associated with poultry diseases or potentially could limit hatchability of fertile eggs by penetration through the egg shell.

Disinfecting Chambers: Four sealable prototype laboratory chambers were constructed of Plexiglas; each measured 39.1 cm x 19.5 cm x 19.5 cm, giving an internal volume of 14.9 L in each chamber (Figure 2.1.27). During the ozonation trials a stainless steel gas manifold allowed

controlled multiexposures of the ozone from the generator to each chamber via individual flow meters set at 0.8 to 0.95 L/min. These same chambers were used in the formaldehyde trials.

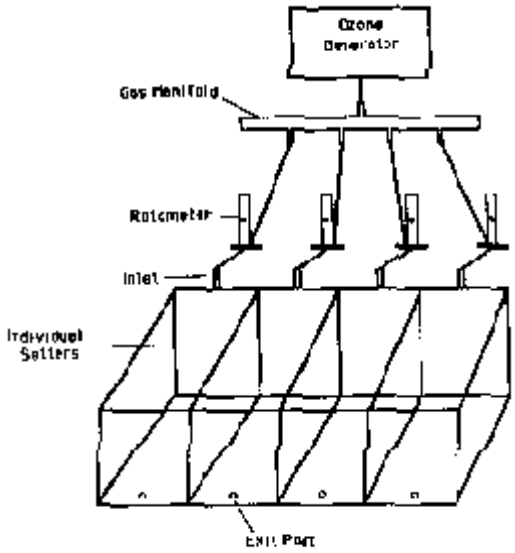


Figure 2.1.27. Laboratory prototype hatchery setter (Whistler and Sheldon, 1989b).

Experimental Protocol: Twenty-four-hour brain heart infusion broth cultures of each bacterium were serially diluted in 0.1% peptone water and spread-plated onto 10 plate count agar (PCA) plates at four different serial dilutions ranging from ca 10^2 to 10^8 depending on the growth characteristics of the bacteria. The initial starting populations of each bacterium were not standardized across replications. Eight uncovered inoculated plates (two per dilution) were placed inside each prototype setter and exposed to either gaseous ozone (1.51 to 1.65% by weight) or triple-strength formaldehyde gas for 0, 2, 4, 6, or 8 min at ambient temperatures (23 to 26EC and 60 to 80% relative humidity. After exposure, the plates were removed, covered, and incubated for 48 h at 37EC. Surviving organisms then were enumerated and the results compared with the nontreated population counts. Spores from four distinct colonies of *A. fumigatus* on mycophil agar were harvested and treated as for bacterial cultures, except that ozonation was at 1.65% by. weight and incubation was at ambient temperature (23 to 26EC for 72 h. Results obtained for all the microbial studies were expressed as geometric means of three experimental replications for each microorganism.

Effect on Cellular Mutation and Defense Mechanisms: Effects of ozone on cell viability and biosynthesis of catalase and superoxide dismutase (SOD) in E. coli (ATCC-MM294) were investigated because colonies of *E. coli* surviving ozonation were much larger than nontreated colonies. Twenty-four hour trypticase soy broth cultures of *E. coli* were serially diluted in 0.1% peptone water, spread-plated onto PCA plates, and ozonated for 2 min as described previously. Surviving organisms were picked for a second ozonation. Surviving microorganisms were recovered and assayed for SOD by the cytochrome c method. Catalase activity was determined by monitoring the disappearance of hydrogen peroxide by spectrophotometry at 240 nm. Protein concentrations in lysed cell suspensions were determined with the Biuret method. The SOD and catalase activities are expressed as units/mg of protein.

Fumigation Studies: Ozone and formaldehyde yielded similar population reductions of *E. coli, P. fluorescens, S. typhimurium* and *Proteus* after 2-min exposure (Figures 2.1.28a,b,c,d). Results of only the highest starting microbial populations are presented in this manuscript, given the consistent kill seen across all starting populations. The formaldehyde caused approximately a 7-$\log_{10}$ reduction for *E. coli, P. fluorescens*, and *Proteus* and an 8 $\log_{10}$ reduction for *S. typhimurium*. **This is compared with ozone gas, which resulted in approximately a 6 log10 reduction for all organisms.** At the end of 8 min the two sanitizers had similar effects, resulting in a 7-$\log_{10}$ or greater reduction to <1 CFU/plate.

The *A. fumigatus* spores were more resistant to ozone gas than vegetative bacteria but their levels were effectively reduced: a >3 $\log_{10}$ reduction with ozone vs. a >5 $\log_{10}$ reduction with formaldehyde after 2 min of exposure, and >4 $\log_{10}$ and >5 $\log_{10}$ reductions, respectively, after 8 min (Figure 2.1.28e).

Staphylococcus, Bacillus, and *Streptococcus* hatchery isolates (Figure 2.1.28f) all were very susceptible to ozonation, with reductions of >4 to 5 $\log_{10}$ after only 2 min of exposure to ozone at 1.59% by weight and >4 to 6 $\log_{10}$ after 8 min. Sensitivity of these isolates to formaldehyde gas was not determined, given the previous uniform reductions of the other microorganisms by the triple-strength formaldehyde. Survival curves after ozonation were biphasic, with rapid reductions seen between 0 and 2 min of fumigation followed by a gradual decline between 2 and 8 min (Figure 2.1.28).

The *E. coli* ATCC 33625 colonies surviving ozonation were much larger than the nonozonated control colonies. Catalase was produced at approximately four times the control concentration, whereas SOD concentrations were lower in the ozonated cells than in the nonozonated cells. However, SOD concentrations were approximately 2 to 3 times higher in this strain of *E. coli* than normally found in most types of bacteria. These findings suggest that the resistance of the *E. coli* survivors to ozone is related to the higher concentrations of catalase and SOD produced by these microorganisms. Colonies that survived the 1x and 2x ozonation continued to display atypical growth when a colony was picked and grown in broth prior to plating. Ozone is a very active microbial mutagen. It is unknown whether this resistance to ozone was induced through mutations by ozone or if the resistance was inherent in a small percentage of the *E. coli* population.

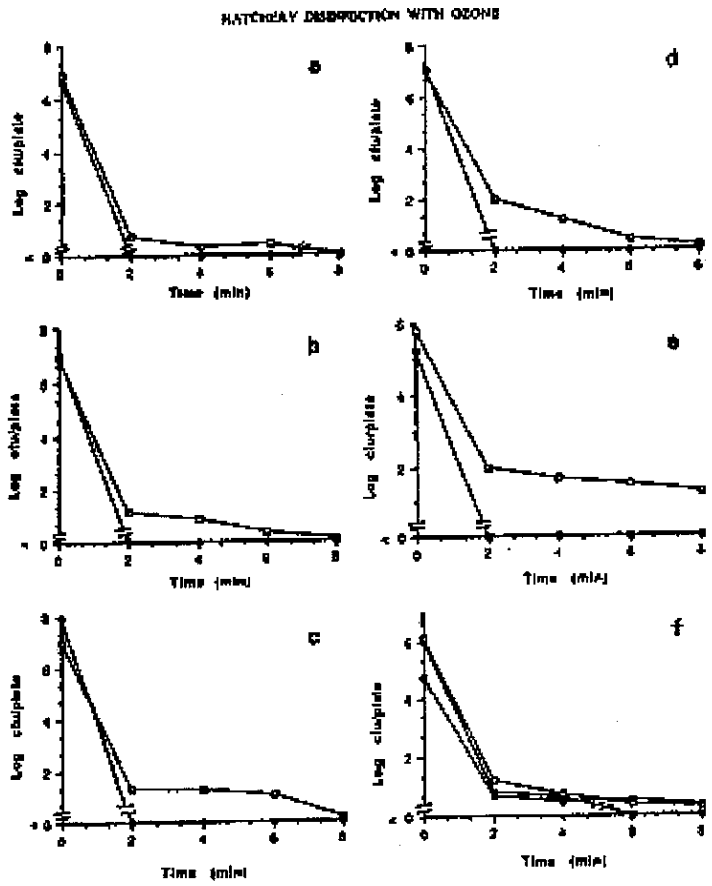


Figure 2.1.28. a. *Escherichia coli* exposure to ozone (1.65% ozone by weight) or formaldehyde (form., triple strength). All points represent geometric means of three replications and have range values of <1 $\log_{10}$; = *E. coli* ozone; = *E. coli* form. b. *Pseudomonas fluorescens* (P. fluor) exposure to ozone (1.65% ozone by weight) or formaldehyde (triple strength). All points represent geometric means of three replications and have range values of <1 $\log_{10}$; = P. fluor ozone; = P. fluor form. c. *Salmonella typhimurium* (S. typhi) exposure to ozone (1.52% ozone by weight) or *formaldehyde triple strength). All points represent geometric means of three replications and have range values* of <1 $\log_{10}$; = S. typhi ozone; = S. typhi form. d. *Proteus* exposure to ozone (1.52% ozone by weight) or formaldehyde (triple strength). All points represent geometric means of three replications and have range values of <1 $\log_{10}$; = proteus ozone; = proteus form. e. . *Aspergillus fumigatus* (A. famig) exposure to ozone (1.65% ozone by weight) or formaldehyde (triple strength). All points represent geometric means of three replications and have range values of <1 $\log_{10}$; = A. fumig ozone; = A. fumig form. f. *Staphylococcus* (Staph), *Bacillus*, and *Streptococcus* (Strep) exposure to ozone (1.59% ozone by weight). All points represent geometric means of three replications and have range values of <1 $\log_{10}$; = Bacillus ozone; X = Staph. Ozone; = Strep. Ozone (Whistler and Sheldon, 1989b).

These preliminary findings illustrate that ozone is an effective alternative disinfectant against some important poultry pathogens and hatchery isolates. Moreover, the microbial reductions seen in this study occurred in the presence of other nonbacteriological organic matter (PCA) that would be expected to increase the ozone demand and lend protection to the organisms. These results support the work of Masaoka et al. (1982), in which ozone fumigation compared favorably with formaldehyde fumigation in decontaminating uninhabited bioclean hospital rooms.

2.1.3.2 Whistler and Sheldon (1989c) – Ozone vs Formaldehyde

Abstract: Ozone and formaldehyde were compared as poultry hatchery disinfectants in a poultry setter, and evaluated for effectiveness. *Escherichia coli, Pseudomonas fluorescens, Salmonella typhimurium* and *Proteus* spp. were inoculated onto open petri plates and exposed to ozone or onto filter paper strips and exposed to ozone or formaldehyde in a poultry setter. Ozone (1.41 to 1.68% by weight) resulted in significant bacterial reductions of >4 $\log_{10}$ on the open plates and >3 $\log_{10}$ on filter paper strips, whereas formaldehyde (triple strength) resulted in >7 $\log_{10}$ reduction on filter paper strips. Ozone similarly was lethal to organisms on filter paper strips at 90% relative humidity (RH) and 13.9EC, and at 50% RH and 37.7EC. Although under the conditions of this study formaldehyde (triple strength) was more lethal than ozone, ozone killed >99.9% of the starting microbial populations. In the event that formaldehyde can no longer be used in the hatchery, an effective alternative may be ozone.

Materials and Methods

Test Organisms: Cultures were obtained from the following sources: *Escherichia coli* ATCC MM294 from the North Carolina State University (NCSU) Food Science Dept. culture collection; *Salmonella typhimurium* B-13, *Pseudomonas fluorescens* B-14, and *Proteus* spp. B-13 from M. Daeschel (USDA Fermentation Laboratory, Dept. of Food Science, NCSU).

Open Plate Fumigation: Twenty-four hour trypticase soy broth cultures of *E. coli* (ca 10/mL), *P. fluorescens* (ca 10^7/mL), *S. typhimurium* (ca 10^6/mL), and *Proteus* spp. (ca 10^6/mL) were serially diluted in 1% peptone water, and spread plated onto plate count agar at four different starting populations. Starting populations were not standardized across replications. Plates were placed uncovered inside a poultry setter (volume: 2.01 m^3) on the top, middle, and bottom egg racks to evaluate possible positional effects. The setter was operated at ambient temperature and 90% RH with the interior air-circulating fan operating and vent open. The 90% RH was chosen, as greater disinfection by ozone normally is achieved at higher RH and moisture levels. Ozone gas entered the setter via Teflon tubing placed through the air vent in the ceiling of the setter, with the end of the tube positioned approximately 30.5 cm from the top of the setter. Plated microorganisms were exposed to ozone gas concentrations ranging from 1.41 to 1.68% by weight at a flow rate of 4.72 L/min. After 37 min of exposure, which correlates with 2 min of exposure in the laboratory prototype setter study of Whistler and Sheldon (1989b), any remaining ozone was removed from the setter by activating, for 10 min, an exhaust fan that was interfaced between the setter and an outside wall vent. Plates were removed, covered, and incubated for 48 h at 37EC along with nontreated controls. Percentage reductions of microbial levels were calculated by subtracting counts of surviving colonies on the exposed plates from the corresponding nontreated controls, dividing the remainder by the control counts, and multiplying

them by 100. Results are expressed as geometric means of three replications for each microorganism. Formaldehyde fumigation was not performed on bacteria in the open petri plates, given the uniform reductions of >7 $\log_{10}$ observed in the previous laboratory studies on the same genera (Whistler and Sheldon, 1989b).

Filter Paper Strip Fumigation: Twenty-four hour trypticase soy broth cultures of *E. coli* (ca 10^6/mL), *P. fluorescens* (ca 10^7/mL), *S. typhimurium* (ca 10^6/mL) and *Proteus* (ca 10^7/mL) were dispersed in 0.05 mL aliquots onto a defined 1 cm x 4 cm section of sterile Whatman #1 filter paper strips. Strips were taped aseptically with uninoculated tabs (in a configuration that did not allow the inoculated section to touch the setter surface) to the top, back, door, and floor of the setter to evaluate possible positional effects. Initial populations were not standardized across replications. The ozonation method used was the same as for the plated organisms, using concentrations of 1.59 to 1.66% by weight ozone at 13.9EC and 90% RH. Additionally, triple-strength formaldehyde fumigation was conducted at 13.9EC and 55% RH with filter paper strips prepared in an identical fashion. After ozonation or formaldehyde exposure for 37 min, the inoculated portion of each strip was aseptically removed, placed individually into 9 mL peptone water, and vortexed to harvest the microorganisms from the strips; serial dilutions were pour plated with PCA. Plates from treated and nontreated strips were incubated for 48 h at 37EC; surviving colonies were enumerated. Results are expressed as geometric means of three replications for each microorganism.

Relative Humidity and Temperature Effects on Fumigation: In order to evaluate the effects of temperature and RH on biocidal rates of ozone and to better reflect actual commercial setter operating conditions, microorganisms were exposed to ozone at 37.7EC and 50% RH for comparison with the treatment described above. The *E. coli* (ca 10^6/mL), *P. fluorescens* (ca 10^6/mL), *S. typhimurium* (ca 10^6/mL), and *Proteus* (ca 10^7/mL) were prepared on filter paper strips as detailed above and ozonated for 37 min at 1.51% by weight ozone. Microorganisms were harvested from the paper strips, incubated, and counted as detailed above.

Open Plate Fumigation: Following ozone fumigation of the inoculated open plates, starting populations of *E. coli* decreased significantly (P<01) to <32 CFU/plate, *P. fluorescens* to <65 CFU/plate, *S. typhimurium* to <32 CFU/plate, and *Proteus* to <32 CFU/plate in all setter locations (Figure 2.1.29). These reductions represent a 4 to 6 $\log_{10}$ kill. Disinfection was uniform across all locations except for *Proteus*, where significant differences in final counts were detected for all incubator locations.

Filter Paper Strip Fumigation: To eliminate the potential protective effect of the PCA on the microbes, the resistance to formaldehyde and ozone of microorganisms inoculated onto filter paper strips was studied. Triple strength formaldehyde significantly (P<01) reduced *E. coli, P. fluorescens, S. typhimurium*, and Proteus populations to <1 CFU/strip or 7 to 8 $\log_{10}$ reductions in all locations within the setter (Figures 2.1.30 and 2.1.31). Ozonation yielded significant reductions of >4 $\log_{10}$ for *E. coli*, >3 $\log_{10}$ for *P. fluorescens* (Figure 2.1.30), >3 $\log_{10}$ for *S. typhimurium*, and >4 $\log_{10}$ for *Proteus* (Figure 2.1.31). Of all the microorganisms tested, *P. fluorescens* exhibited significantly (P<0.01) more resistance to ozone than formaldehyde. No consistent microbial reduction pattern was detected across the four sample locations, indicating uniform dispersion of the two disinfectants.

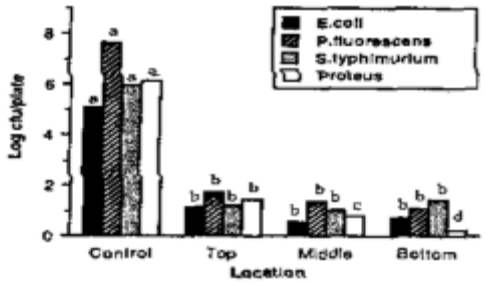


Figure 2.1.29. Inactivation by ozone [1.41 to 1.68% by weight, 15.6EC, 90% relative humidity (RH)] of selected microorganisms on the surface of agar medium in petri plates located at several positions in a setter. Height of bars represents geometric means of three replications; bars have range values of <1 $\log_{10}$. Values within bacterial species with no common letters are significantly different (P<.01). E. = *Escherichia*; P. = *Pseudomonas*; S. = *Salmonella* (Whistler and Sheldon, 1989c).

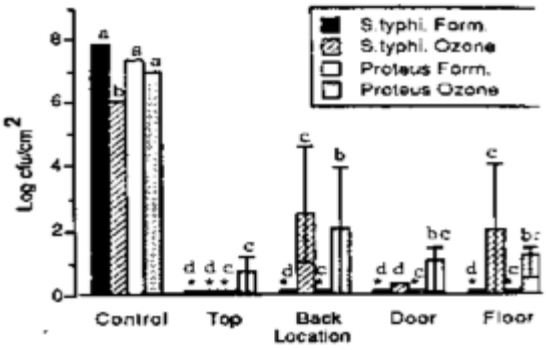


Figure 2.1.30. Inactivation by ozone [1.59 to 1.66% by weight, 13.9EC, 90% relative humidity (RH)] or formaldehyde (Form., triple strength; 13.9 C/55% RH) on *Escherichia coli* (*E. coli*) and *Pseudomonas fluorescens* (P. fluor.) inoculated onto filter paper strips, located at several setter positions. *Values are <1 CFU/cm^2. Height of bars represents geometric mean of three replications; bars have range values of <1 $\log_{10}$ unless otherwise indicated by range bars (I). Values within bacterial species with no common letters are significantly different (P<0.01) (Whistler and Sheldon, 1989c).

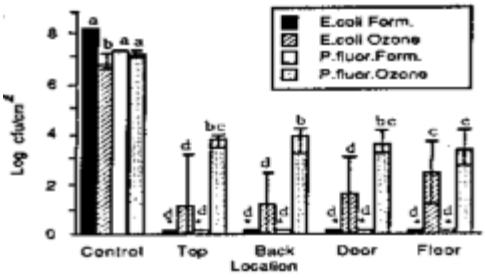


Figure 2.1.31. Inactivation by ozone [1.59 to 1.66% by weight, 13.9EC. 90% relative humidity (RH)] or formaldehyde (Form., triple strength; 13.9 C/55% RH) on *Salmonella typhimurium* (S. typhi.) and *Proteus* inoculated onto filter paper strips located at several setter positions. *Values are <1 CFU/cm_2. Height of bars represents geometric means of three replications; bars have range values of <1 log_{10} unless otherwise indicated by range bars (1). Values within bacterial species with no common letters are significantly different (P<0.01) (Whistler and Sheldon, 1989c).

Relative Humidity and Temperature Effects on Fumigation: *Pseudomonas fluorescens* displayed significantly (P<0.01) more resistance to ozone at high humidity (90% RH), with around 3 log_{10} reductions detected compared to 4 log_{10} reductions at low humidity (Figure 2.1.32). Although counts of *E. coli* and *S. typhimurium* in response to ozone treatment were significantly reduced by >4 log_{10} and *Proteus* by >5 log_{10} at all setter locations, the effect of humidity was inconsistent (Figures 2.1.32 and 2.1.33).

Discussion: Ozone gas at concentrations of 1.56% by weight killed more than 99.9% of the four starting bacterial populations inoculated onto either PCA petri plates or filter paper strips. These results support the findings of other disinfection studies where ozone was used to disinfect wastewater and hospital rooms. Exposure of the microorganisms to ozone on either PCA or paper strips resulted in similar kills. Thus, the protective effect provided by the culture medium for the plated microorganisms during ozonation was not observed. The efficacy of most disinfectants generally is inversely related to the level of non-microbiological organic matter present in the system. The ability of ozone to kill bacteria inoculated onto PCA, as observed in this study, is encouraging from a hatchery disinfection standpoint, as most incubators and hatching eggs are contaminated with considerable nonmicrobiological organic matter. Furthermore, starting bacterial concentrations used in this study are much higher than normally found in hatcheries. These factors together indicate that ozone would be effective under hatchery conditions.

Ozone appeared to be evenly dispersed throughout the incubator as indicated by the consistent bacterial kill seen at each sample location. No consistent RH effects other than for *P. fluorescens* were observed in the ozonation studies. The increased resistance of *P. fluorescens* at 90% RH is consistent with the fact that this organism is prevalent on poultry farms experiencing problems with high moisture levels in the layer houses.

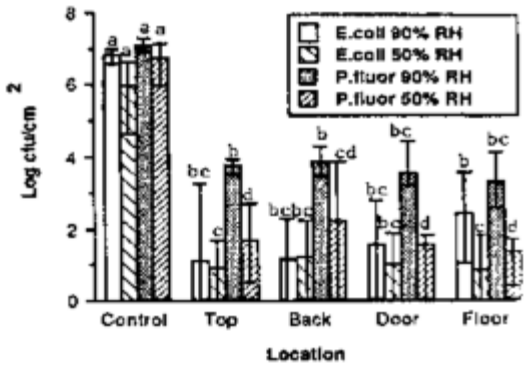


Figure 2.1.32. Inactivation by ozone [1.59 to 1.66% by weight, 13.9EC, 90% relative humidity (RH) or 1.51% by weight, 37.7EC, 50% RH] on *Escherichia coli* (*E. coli*) and *Pseudomonas fluorescens* (P. fluor.) inoculated onto filter paper strips located at several setter positions. Height of bars represents geometric means of three replications; bars have range values of <1 $\log_{10}$ unless otherwise indicated by range bars (I). Values within bacterial species with no common letters are significantly different (P<0.01) (Whistler and Sheldon, 1989c).

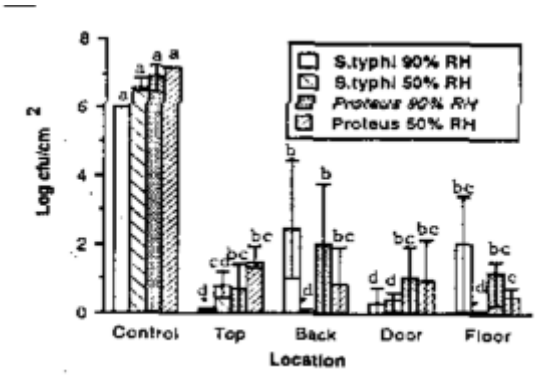


Figure 2.1.33. Inactivation by ozone [1.59 to 1.66% by weight, 13.9EC. 90% relative humidity (RH) or 1.51% by weight, 37.7EC, 50% RH] on *Salmonella typhimurium* (S. typhi.) and *Proteus* inoculated onto filter paper strips located at several setter positions. *Values are <1 CFU/cm^2. Height of bars represents geometric means of three replications; bars have range values of <1 $\log_{10}$ unless otherwise indicated by range bars (I). Values within bacterial species with no common letters are significantly different (P<0.01) (Whistler and Sheldon, 1989c).

The procedure to generate ozone could be easily automated and was more controllable than that of formaldehyde, which required hand measuring and mixing of hazardous chemicals. Furthermore, the output of ozone from the generator can be constantly regulated and monitored using commercially available ultraviolet ozone detectors. Also, these monitors can be used to shut down the generation system should an ozone leak occur in surrounding hatchery areas. Finally, another safety advantage of ozone is that it is detected (smelled) at concentrations (0.01 to 0.05 ppm by volume), much lower than reported toxic levels.

SUMMARY -- Poultry -- (Meat, Chiller Water and Studies with Ozone in Air)

Numerous studies by a variety of investigators have demonstrated the microbiocidal efficacy and safety of ozone for use in washing poultry carcasses, reconditioning poultry chiller water, and sanitizing hatchery equipment. Advanced Oxidation Processes (AOPs), including ozone and adjuncts such as hydrogen peroxide and UV radiation, have been shown to enhance the efficacy of ozone as an antimicrobial control agent in certain instances. Effective prefiltration of chiller water prior to ozone treatment is recommended for optimum reduction of microbiological levels and efficient use of ozone.

Data collected from studies published during 1979 through 1999 are cited in this section of the FAP, showing clearly that poultry carcasses can be sanitized and poultry chiller water can be reconditioned for reuse safely and efficiently, well within current U.S. Dept. of Agriculture guidelines, with no sensory degradation, buildup of pathogens, other spoilage microbes, or production of toxic oxidation byproducts.

2.2 RED MEAT PROCESSING

2.2.1 Kaess and Weidemann (1968) – Beef Muscle Slices

Summary: The effects of continuous ozone treatment at 0.3EC on the growth of psychrophilic meat spoilage organisms, grown on muscle slices with equilibrium relative humidity (EH) of 99.3, 98.5 and 98.0% were investigated. Ozone concentrations ranged from 0.13 to 5.0 mg/m^3 in air and the microorganisms included pigmented and non-pigmented *Pseudomonas*, the yeast *Candida scottii*, and the molds *Thamnidium* and *Penicillium*. A treatment at EH 99.3% with air containing carbon dioxide (11%) and ozone (0.6 mg/m^3) was included.

Small but significant inhibitory effects on non-pigmented *Pseudomonas* species and on the yeast *Candida scottii* were obtained with ozone concentrations $\exists$ 2 mg/m^3 (EH 99.3%). A concentration of 0.6 mg/m^3 was not always significantly effective with non-pigmented *Pseudomonas* species, but significant decreases were recorded for the slower growing pigmented *Pseudomonas* species.

With ozone concentrations $\exists$0.6 mg/m^3, the population density of 10^8 cells/cm^2, at which bacterial colonies first become manifest in air (slime point), was increased to about 10^9/cm^2. Lowering the EH or introducing 11% carbon dioxide into the storage further delayed the appearance of the "slime point" of the bacterial population owing to ozone treatment.

In the presence of ozone, the lag phase of *Thamnidium* and *Penicillium* was longer but the growth rate was about the same as that of the controls. No aerial mycelium appeared with ozone concentrations $\exists$0.6 mg/m^3.

The color of the treated muscle surface did not differ from that of controls when the ozone concentration of the storage atmosphere was # 0.6 mg/m^3.

In the present experiments a continuous ozone concentration was decreased in steps to find the effect on growth of microorganisms at which color changes of chilled meat remained negligible during storage.

Methods

Sample Preparation: Meat samples were obtained from fresh semitendinosus muscles under sterile conditions. Circular discs with a diameter of 7.6 cm were cut with a cutting cylinder from samples 0.12 cm. thick, sliced from the muscle perpendicular to the fiber direction, with a mechanical cutter. The slices fitted sterile, stainless steel sample holders which exposed a meat area of 35 cm^2/side to the ozone.

Equilibrium relative humidity (EH) of 98.3 and 98.0% over the slices was obtained by exposing them, in a duct, to an air stream flowing at a rate of 3.5 m/sec at 2EC and 80% RH, to reduce the moisture content to the values for a definite EH.

Apparatus: A continuous stream of air (30 L/h) with EH of 99.3, 98.5 or 98.0% and a constant temperature of 0.3EC was drawn through the containers. The ozone concentrations ranged from

0.15 to 5 mg/m^3. In two experiments the effect of a concentration of 0.6 mg/m^3 ozone in a mixture of air with 11% CO_2 was tested. The EH was maintained by passing the dry air through distilled water in a gas wash bottle and then through a glass coil (20 ft long, 0.5 inch inner diameter) filled with sulfuric acid by submerging it in a desiccator with the solution having the concentration to establish the EH.

Microbiological Procedures: Diluted cultures of typical meat spoilage, non-pigmented (Nos. 1482, 131, 39 and A2) and pigmented (Nos. 221, 91 and 41) *Pseudomonas* species, grown for 3 days in nutrient broth at 23EC, were sprayed on the meat samples in the holders. Suspensions of the yeast *Candida scottii*, grown in Wickerham's Y.M. broth, were blended in the Buehler homogenizer for 1 min, diluted in 0.5% peptone water and filtered before spraying. Samples were taken with sterile cutting cylinders from each of the two slices used for an experiment and the population determined by the poured plate method. Bacteria were plated on nutrient agar and yeasts on potato dextrose agar. Incubation was for 5 days at 20EC.

Spores were washed off slope cultures of *Thamnidium* or *Penicillium* species with a small quantity of physiological saline, to which a drop of non-ionic detergent had been added as wetting agent. This suspension of spores was used to inoculate meat samples in the center with the ground end of a sterile glass rod.

Histological Examination: Pieces of about 1 cm^2 were cut from meat slices and fixed at room temperature by placing the side free of organisms on top of several layers of filter paper, the lower layers of which were submerged in 10% formalin solution. With this method losses of microbial colonies were avoided. After fixation the samples were dehydrated in an alcohol series, embedded in paraffin and sectioned at a thickness of 10 Φ.

Sections of samples with bacterial colonies were stained with Gram stain; sections with mold were overstained with haematoxylin and cosin, and both examined with the light microscope.

Statistical Analysis: The significance of the effect of ozone on bacterial growth was tested by examining the difference between the logarithms of counts of treated and control samples at the middle of the time range and the extent to which the difference remained significant. when averaged over increasing ranges of time. The significance of differences was given in all cases for a probability level of 5%.

Results

Effects of ozone on growth of bacteria: At 0.3EC and 99.3% EH, ozone concentrations of 0.15-5 mg/m^3 delayed moderately the growth of non-pigmented *Pseudomonas* species (Figure 2.2.01). In the prese4nce of ozone, the lag phase was increased but the growth rate was not affected. Differences in population density between *Pseudomonas* 131, 1482 and A_2 exposed to ozone concentrations 5 or 2 mg/m^3 and controls, as shown in Figure 2.2.01 were significant, except for organisms 1482, for which treatment (5 mg/m^3) was significant only towards the end of the storage time. With the low concentration of 0.6 mg/m^3, differences between treated and untreated samples were secured for organism 1482 but not for 131. The bactericidal effect practically disappeared with an ozone concentration of 0.15 mg/m^3.

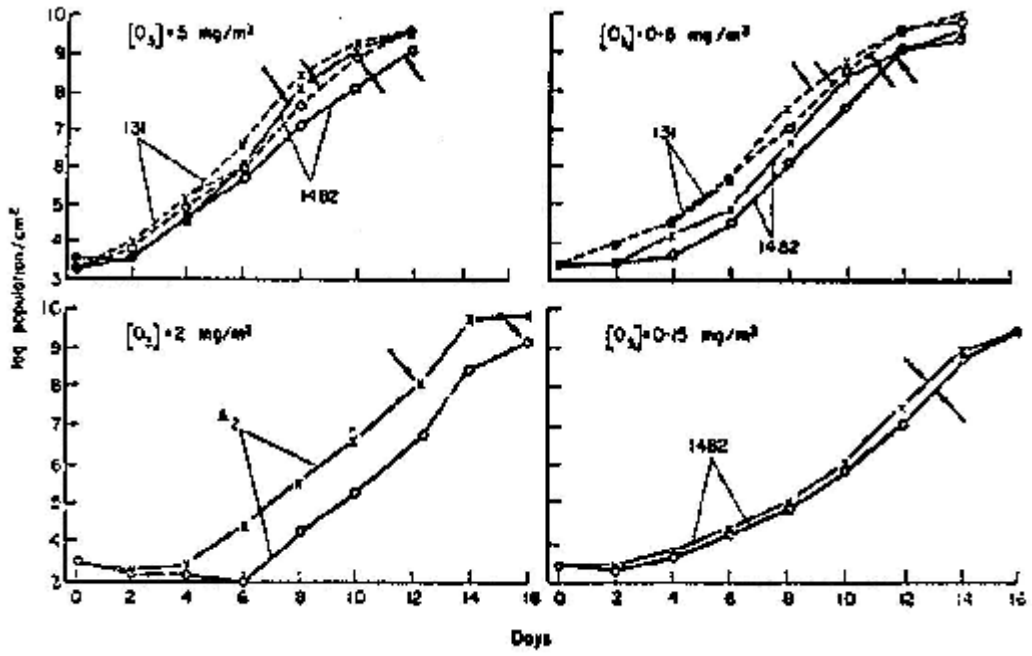


Figure 2.2.01. Logarithm-time growth curves for non-pigmented *Pseudomonas* species 131, 1482 and A_2. Organisms growing on slices of *Musculus semitendinosus* in air or in air-ozone mixtures at 0.3EC and 99.3% EH. Ozone concentrations of 0.15-5 mg/m^3. The appearance of slime point is indicated by arrows. " = Ozone; X = air (Kaess and Weidemann, 1968).

The retardation of growth of pigmented *Pseudomonas* species was significant, mainly due to an extended lag phase. In some cases the variance between ranges or within ranges of growth curves was significant, caused probably by the variation in composition of the muscle (connective tissue, fat content) and also by the variation of ozone absorption within and between slices.

The time at which bacterial colonies first became manifest in air at a population density of 10^8 organisms/cm^2 was delayed until the population increased to approximately 10^9/cm^2 (Figures 2.2.01 and 2.2.02) with concentrations of ozone $\exists$ 0.6 mg/m^3.

Histological examination showed that at the time when colonies of bacteria growing in air had merged into a film, the surface of muscle exposed to an ozone concentration of 5 mg/m^3 was free of organisms and colonies were situated predominantly in crevices of the tissue and openings of vessels, where they were protected from the direct effects of ozone. At lower concentrations of ozone, individual colonies developed leaving sterile areas of muscle between them. The distinct dark layer, observed on the muscle surface after storage for 12 days at an ozone concentration of 5 mg/m^3 was absent on tissue stored at lower concentrations.

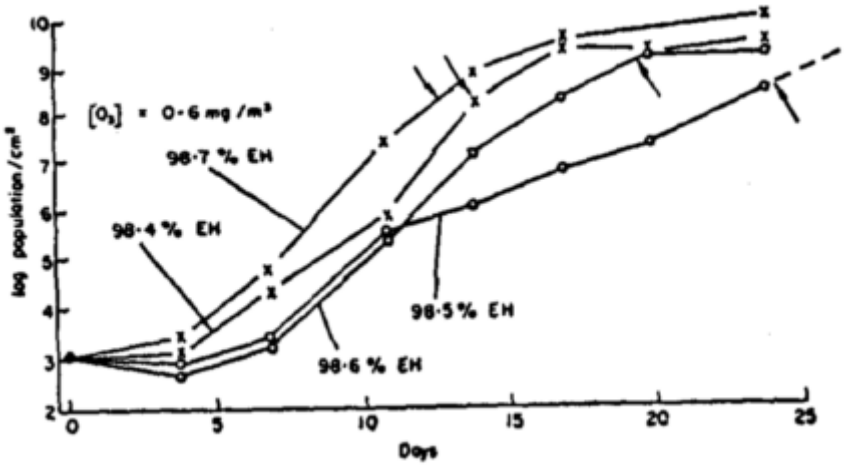


Figure 2.2.02. Logarithm-time growth curves for *Pseudomonas* 1482 growing on slices of *M. semitendinosus* with an EH of approximately 98.5% (exact value indicated on curve) in air and air-ozone mixture of 98.5% EH at 0.3EC. Each curve represents growth on an individual slice. Slime point is indicated by arrows. " = Ozone; X = air (Kaess and Weidemann, 1968).

Influence of Equilibrium Relative Humidity: Figure 2.2.02 shows the maximum inhibitory effect of ozone (0.6 mg/m^3) obtained with the non-pigmented *Pseudomonas* 1482 at an EH of 98.5%. The retarding effects of the ozone were significant, but differences were smaller and insignificant in another experiment. The slime point again was delayed until the population increased to 10^9 cells/cm^2. With an EH of 98.0% (moisture content 150% dry weight), growth of *Pseudomonas* 1482 in air was prevented. In a mixture of air and ozone (0.6 mg/m^3) the population declined slowly and approximately linearly (0.035 log population/day).

Bacterial Growth in Air-Carbon Dioxide-Ozone Mixture: Experiments with *Pseudomonas* species exposed to an atmosphere with 11% CO_2 at 0.3EC confirmed earlier results of Scott (1938) which showed that the growth rate of bacteria could be reduced by 50-60% by addition of 10% CO_2 to the air. *Pseudomonas* 131 was the most sensitive to CO_2 of the species tested. With this markedly reduced growth rate, the effect of adding 0.6 mg/m^3 ozone to the air-carbon dioxide mixture was significant only at the end of the experiment for *Pseudomonas* 1482 and there was no increased effect for *Pseudomonas* 131. Colonies were visible at a cell density of 10^8/cm^2 when growing in air with 11% CO_2, but the slime point appeared at a density of about 10^9/cm^2 in an atmosphere with 11% CO_2 and 0.6 mg/m^3 ozone.

Effect of Ozone on the Growth of Yeasts: The growth of *Candida scottii* on muscle slices kept at 0.3EC and 99.3% EH was retarded significantly in air with 5 mg/m^3 and 2 mg/m^3 ozone, but the effect was insignificant in air with 0.6 mg/m^3 ozone. Instead of the moist appearance (slime point) of control samples, a dry dull surface was apparent on treated samples.

Effect of Ozone on the Growth of Molds: The lag phase of *Thamnidium* and of *Penicillium* was increased by about two days over that of the controls (Figure 2.2.03) when the molds developed on muscle stored in air with ozone concentrations of 0.16-5.0 mg/m^3. The growth rate of

Thamnidium was slightly but significantly smaller than controls with ozone concentrations of 5 and 1.9 mg/m^3, but not with smaller concentrations. *Penicillium* had a tendency to grow at a slightly higher rate in ozone treated muscle than in controls.

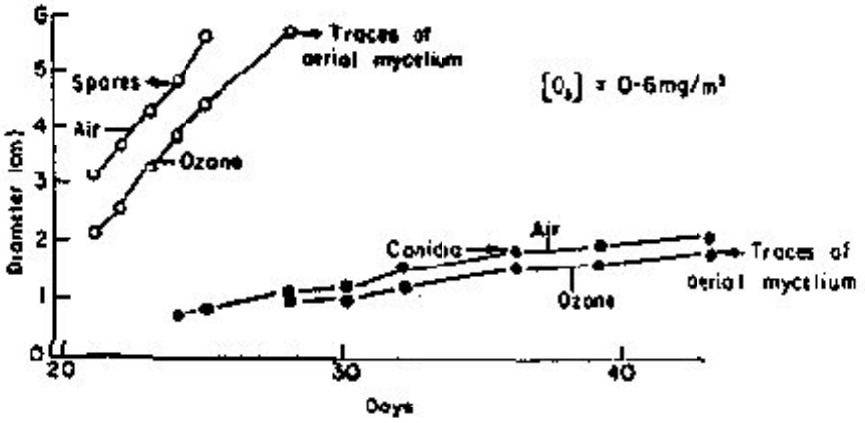


Figure 2.2.03. Increase with time of colony diameter of *Thamnidium* (") and *Penicillium* (!) growing on slices of *M. semitendinosus* in air and air with 0.6 mg/m^3 ozone at 0.3EC and 99.3% EH (Kaess and Weidemann, 1968).

Normal aerial mycelium did not develop on mold growing in air with ozone at concentrations ∃ 0.6 mg/m^3. At a concentration of 0.6 mg/m^3, traces of mycelium were just visible on the muscle slices at the end of the experiment. At an ozone concentration of 0.16 mg/m^3 thickened sterile aerial hyphae of *Thamnidium* grew to a length of 1-2 mm (controls about 10 mm). *Penicillium* hyphae had a length of a fraction of 1 mm.

Histological examination showed that *Penicillium* exposed to 1.9 mg/m^3 ozone developed freely between and within muscle fibers. Aerial mycelium with conidia appeared on the control sample. Similar results were obtained with *Thamnidium* but short, single, thickened aerial hyphae showed up on the muscle surface in an atmosphere with 1.9 mg/m^2 ozone.

Effects of Ozone on Color of Meat: The meat pigments myoglobin and hemoglobin were quickly oxidized to brown heme compounds in an ozone concentration of 2 mg/m^3 or more, but at a level of 0.6 mg/m^3 there was no difference in color between samples and controls when the storage life was terminated at population densities of 10^9 and 10^8 cells/cm^2, respectively. Short times between slaughter and the beginning of cooling and treatment, and also high pH values (close to 6) tended to prolong storage times for the onset of discoloration.

Discussion: The need to prevent discoloration of the muscle surface sets low limits to the permissible concentration or a continuous ozone treatment. The absence of a linear relationship between the duration of treatment to produce a brown discoloration, and the reciprocal ozone

concentration excludes using the Bunsen-Roscoe law for the calculation of the concentration-time relationship of discoloration.

The effect on growth of microorganisms of ozone at concentrations which did not noticeably cause discoloration of the muscle varied with organisms. The reduction of growth was greatest with naturally slow growing pigmented *Pseudomonas* species, but only small when the growth rates of rapidly growing *Pseudomonas* 1482 and 131 were artificially reduced by decreasing the EH of the muscle or by using an atmosphere with 11% CO_2. The retarding effect of ozone(0.6 mg/m^3) at an EH of 98.5% on growth of *Pseudomonas* was of the same order as that obtained with control samples whose moisture content (% dry weight) was lowered by 20%, i.e., at an EH of 98.3%. The increased inhibition of bacterial growth due to ozone (up to 10 mg/m^3, 3 hr/day) with decreasing EH of muscle, was not observed with a continuously applied low ozone concentration (0.6 mg/m^3).

Ozone was mainly effective on organisms directly exposed to concentrations ∃ 0.6 mg/m^3. Bacterial growth appeared to be strongly reduced in the distinct dark layer on muscle exposed to an ozone concentration of 5 mg/m^3. This inhibition probably was due to oxidation of tissue close to the surface. As a consequence of this, bacteria started growth in crevices under the surface, formed isolated colonies, and the appearance of the slime point -was delayed. At ozone concentrations ∃ 0.6 mg/m^3 of *Thamnidium* and *Penicillium* developed mycelium almost entirely in the substrate.

Optimal conditions for muscle stored at 0.3EC were obtained by applying a continuous ozone concentration of 0.6 mg/m^3.

2.2.2 Greer and Jones (1989) – Beef Carcasses

Abstract: A study was undertaken, using experimental slaughter and dressing conditions, to determine the effects of ozone (0.03 ppm) upon beef carcass shrinkage, carcass characteristics, muscle quality and total mesophilic and psychrotrophic bacteria. Paired sides were either continuously ozonated using a commercial ozone generator or subjected to conventional air chilling under identical conditions of humidity (95%) and temperature (1.6EC) for up to nine days of aging. After aging, control and ozone-treated sides were processed and bacterial growth and retail case life determined for steaks on simulated, retail display. Cooler shrinkage over nine days was significantly higher (10.6 g/kg) in ozone-treated compared to control sides and this difference between treatments increased (14.9 g/kg) following trimming of discolored and dry muscle tissue. Although ozone prevented bacterial growth on carcass surfaces, it did not affect the retail case life (odor, appearance) or reduce bacterial growth on retail steaks. Ozone had the deleterious effects of dramatically increasing shrinkage weight losses and trim losses and darkening lean muscle color.

Materials and Methods

Treatments: Beef carcass aging was conducted in identical meat coolers (11.5 m long, 3.2 m wide, 5.1 m high) with cooling units operating at 7.47 x 10^{10} joules/h and 266 kL/min. Under these conditions, air velocity was 0.5 m/s, cooler temperature was 1.6 + 0.2EC and the temperature measured at the carcass surface (after 24h of chilling) was 2.6 ∀ 0. 1EC. Relative

humidity was monitored using a continuous recording hygrothermograph and found to be identical in both control and ozonated coolers (95%).

An Ozone Air Sterilizer was installed. On the day of installation and following 6 d of carcass aging, cooler levels of ozone were determined by drawing air into Gastec Detector Tubes using a piston type volumetric pump. Thirty, 100 mL air samples were taken at each sampling interval at several locations throughout the cooler. Ozone concentration remained constant at 0.03 ppm. An identical cooler, in the absence of ozone. served as control.

After slaughter, left and right sides of 10 beef carcasses from crossbred beef animals (average live weight = 450 kg) were chilled for 24 h at 10EC before randomly assigning alternate sides to the ozone or control treatments. This approach was taken to obtain more realistic bacterial levels on the carcass surface. Carcass quality measurements and bacterial samples were taken before and after 9 d of carcass aging at 1.6EC.

Case Life Studies: Following carcass aging for 9 d, six rib-eye steaks were fabricated from each carcass side subjected to each treatment. To minimize the effects of contamination during cutting and to standardize the procedure for both treatments, steaks were processed on cutting tables rinsed with pressurized 80EC water and sanitized with Foam-Eze immediately prior to processing carcasses. Cutting knives were cleaned before cutting each rib by immersion in 80EC water for 30 s. Carcasses subjected to each treatment were segregated and processed separately to avoid cross-contamination during processing.

Steaks were wrapped in an oxygen-permeable (2185 cc/m^2/24 h) polyvinyl chloride film and randomly placed in a horizontal retail case under laboratory-simulated retail conditions (50 steaks/treatment). The remaining 10 steaks/treatment were immediately examined to determine initial bacterial densities, appearance and odor. On days 2, 4, 6, 8 and 10 of retail display, an experienced five-member sensory panel for odor and appearance evaluated 10 steaks from each treatment. The same 10 steaks (at each sampling time) were examined for bacterial content as described below.

Bacterial Analyses: Sides were sampled immediately before and immediately following aging by aseptically excising 10 cm^2 areas at three locations (chuck, plate, hip). Following homogenization (Stomacher 400) and decimal dilution (0.1% peptone - water), 0.1 mL amounts were surface plated in duplicate on plate count agar (Difco). One set of plates was incubated at 35EC for 48 h to enumerate mesophilic bacteria and a duplicate set at 7EC for 7 d to enumerate psychrotrophic bacteria. Bacterial numbers on steaks during retail display were determined in a similar fashion following the excision of 10 cm^2 of tissue from the surface of each steak. At each sampling time, the same steaks were used for both bacterial analyses and visual evaluation.

Statistical Analyses: All shrinkage, meat quality, bacterial counts and shelf life data were analyzed by analysis of variance. The effects of ozone on shrinkage weight losses and meat quality was performed using a one-way analysis of variance. Differences in bacterial numbers were evaluated by two-way analysis of variance. Classification variables included number of days of retail display at the time of sampling, aging treatment and appropriate interaction. Differences in retail case life were evaluated in an identical fashion. The significance of

differences between means were determined using the Student's 't' test. Results were considered significant if the calculated probability was less than 0.05.

Carcass Contamination: The data in Tables 2.2.01 (psychrotrophs) and 2.2.02 (mesophiles) compare the effects of ozone and control treatments upon quantitative changes in bacterial densities after 9 d of carcass aging. Results were similar for either bacterial group. Prior to the aging treatment, there were no significant ($P > 0.1$) differences in the numbers of bacteria on carcasses. After 9 d of carcass aging, however, bacterial numbers on control carcass sides were about 10-fold greater than those on ozone-treated carcasses ($P < 0.05$). This difference was found since bacterial populations increased significantly ($P < 0.02$) on control carcasses during 10 d of aging but remained essentially the same on ozone-treated carcasses ($P > 0.1$).

Table 2.2.01. Effects of ozone on psychrotrophic bacteria on beef carcasses [1]
(Greer and Jones, 1989)

Sample time	Log bacteria/cm^2		
	Control	Ozone	P
Before aging	2.87	2.81	0.892
After 9 days aging	4.03	2.83	0.011
P	0.013	0.957	
[1] Least square means of ten sides. The standard error was 0.32.			

Bacterial Growth on Steaks: The effects of ozone treatment of carcasses on the growth of psychrotrophs (Figure 2.2.04) on retail rib-eye steaks was compared to untreated control samples for up to 10 d of retail display. Since similar results were obtained for the mesophilic bacterial population, the growth of psychrotrophic bacteria, only, was chosen for the purposes of illustration. Bacterial numbers on steaks derived from control or ozone-treated carcasses were not significantly different ($P > 0.1$) on days 0, 2 or 4 of retail display. However, bacterial densities when compared to control samples were significantly ($P < 0.01$) greater on steaks derived from ozone-treated carcasses after 6, 8 and 10 days of retail display.

Table 2.2.02. Effects of ozone on mesophilic bacteria on beef carcasses [1]
(Greer and Jones, 1989)

Sample time	Log bacteria/cm^2		
	Control	Ozone	P
Before aging	2.95	2.90	0.912
After 9 days aging	3.95	3.10	0.043
P	0.018	0.621	
[1] Least square means of ten sides. The standard error was 0.29.			

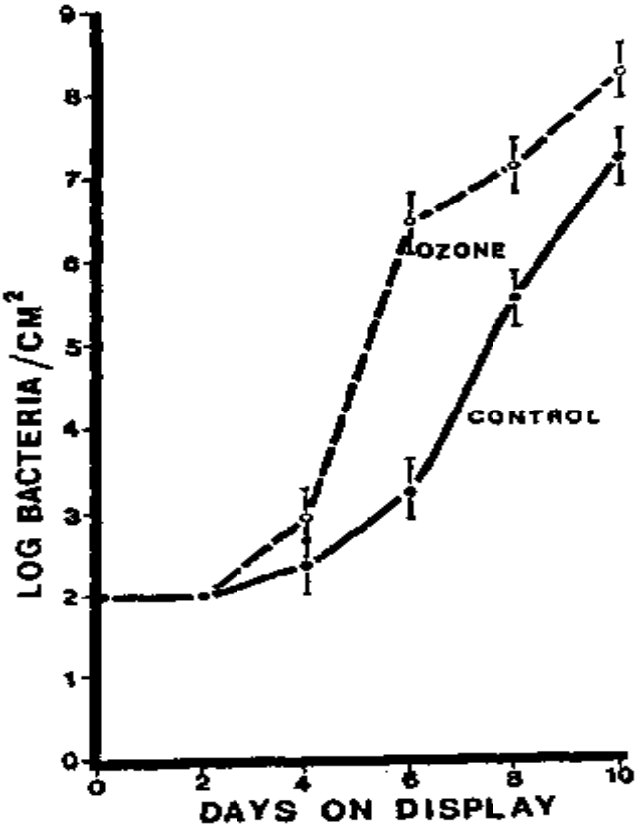


Figure 2.2.04. Effect of ozone on the growth of psychrotrophic bacteria during the retail display of beef rib-eye steaks. Each point represents the least squares mean (Λ SE) for 10 steaks (Greer and Jones, 1989).

The data in Figure 2.2.04 would suggest that differences in bacterial numbers were not due to differences in bacterial growth rates, but rather due to a slightly extended lag phase for bacterial growth on control steaks.

Conclusions: The current study constitutes the first known report of the effects of ozone during the aging of carcass beef. The results showed that although ozonization prevented bacterial growth on carcass surfaces during 9 d of cooler aging, it did not improve the bacterial condition, nor the keeping quality of retail beef steaks derived from ozone-treated carcasses. The inhibition of bacterial growth on carcass surfaces cannot be unequivocally attributed to ozone. That is, evaporative losses from ozone-treated carcasses were significantly greater than those of untreated control carcasses and surface dessication is known to limit bacterial growth. The increased moisture losses from ozone-treated carcasses may have been due to the deterioration of surface tissues (Kaess and Weidemann, 1968a) although this contention remains speculative.

Surprisingly, bacteria were able to initiate growth more rapidly on retail steaks derived from ozone-treated carcasses. Relative to this, the lag phase of bacterial growth was somewhat shorter and consequently, bacterial densities were substantially greater on steaks from ozone-treated carcasses when compared to untreated control samples (after 6, 8 and 10 d of retail display).

Although this result may have been artifactual, it is conceivable that ozone selected for a bacterial population with the potential to initiate growth more rapidly at muscle surfaces.

The current study used ozone at concentrations of 0.03 ppm which is considerably less than the 3.88 ppm found to be effective in delaying poultry spoilage (Yang and Chen, 1979). Contrarily, it has been reported that even 500 ppm ozone had no effect on the microflora of refrigerated beef (Yang and Chen, 1979).

Ozonization of carcasses for up to 9 d of aging did not improve the keeping quality or bacterial condition of retail steaks. Ozone treatment did, however, significantly prevent the growth of organisms on carcass surfaces. This latter benefit undoubtedly would be offset by the substantial shrink and trim losses promoted by ozone.

2.2.3 Mitsuda et al. (1990) – Ozone + CO_2 with Beef

Abstract: The synergistic effect of ozone and carbon dioxide gases on the sterilization of food was investigated. Raw beef and an agar plate of *Escherichia coli* were sterilized with a mixture of ozone and carbon dioxide gases in polyvinyl chloride film bags and stored. In both direct sterilization and the storage tests, the survival percentages for the mixed gases of ozone and carbon dioxide were lower than for those of the individual gases.

The reasons for this synergistic effect were considered to be that the bactericidal effect of ozone gas was retained during the storage period by the quenching effect of carbon dioxide gas to the chain reaction of ozone degradation, and by the bacteriostatic effect of carbon dioxide gas. The mixture of ozone and carbon dioxide gases sterilized both the surface and the inside of the food and the agar plate at the same time.

Materials and Methods: Raw beef marbled with fat was purchased from a local market, sliced to a thickness of 0.7-1.0 cm (about 20 g), and each slice packed in a bag made of polyvinyl chloride. The organisms (a representative strain which behaved biochemically as a typical member of *Escherichia coli*) were cultured in it medium comprising a standard agar for bacterial growth at 37EC for 48 hours.

Preparation of the Mixed Gas: The ozone gas was generated using commercial grade oxygen as the gas supply. The concentration of ozone gas generated in this apparatus was estimated to be 20-40 $g/m^{3.}$ Mixtures of ozone and carbon dioxide gases were prepared with a gas mixing tool by regulating the pressure of the gases to ratios of 3:1, 2:1, 1:1, 1:2 and 1 kg/cm^2 ozone (oxygen): 3 kg/cm^2 carbon dioxide.

Sterilization and Storage of Foods: In the food sterilization process, a bag of 20 cm x 1:1 cm, which had been made of a polyvinyl chloride film having no air-permeability, was used. After opening, each bag which contained the food was 80% sealed. The food in each bag had been exposed for 5 minutes to ozone gas, carbon dioxide gas or their mixture. Then the bag was completely sealed. Microbiological tests were carried out just after the 5-minute sterilization, and later after storage for 7 or 14 days in a refrigerator.

Microbiological Test for Sterilized Foods: Agar plate samples were prepared in accordance with a method of mixing and dilution. Twenty grams of sliced raw beef meat were homogenized

with 100 mL of physiological saline in an homogenizer. One mL of liquid was mixed with 10 mL of agar culture medium and then incubated at 37EC for 48 h. Each test was carried out in triplicate.

Sterilization of Bacterial Cells in the Agar Plate: To sterilize bacterial cells of *Escherichia coli*, plates containing bacterial cells were prepared by mixing one mL of properly diluted cells, which had been precultured in a brain heart infusion broth and 15 mL of agar culture medium in a glass Petri plate of 9 cm diameter. The sterilizing method was the same as in the case of the food described above. After opening, each 20 x 13 cm bag was 80% sealed. The plate in each bag had been exposed for 5 minutes to ozone gas, carbon dioxide gas or their mixture. Just after the 5-minute sterilization, plates were taken out of the bags and then incubated at 37EC for 48 h. For the storage tests, the bags were completely sealed after the 5-minute sterilization and the plates were stored in the same gas at 37EC for 48 hrs. Calculations of the amount of bacteria in these plates were carried out after 48 hrs.

Results and Discussion

Sterilization of Raw Beef: As shown in Table 2.2.03, a large number of bacteria remained in the raw beef even after 5 minutes of sterilization. The lethal threshold concentration for the bacterial cells was reported to be 0.1-0.2 mg/liter when the bacterial cells were bubbling with ozone in a test solution. A low concentration of ozone was reported to be ineffective when organic matter was present, because the organic matter interferes with the action of the ozone on the bacterial cells. Ozone also was reported to kill all bacteria immediately at a critical concentration known as the "all-or-nothing" response." So these results indicate that the organic matter present in the beef samples exerted an ozone demand and prevented the full utilization of the applied dose as a disinfectant.

Table 2.2.03. Survival of bacteria in different gases and gas mixtures just after exposure of sliced beef meat at room temperatures for 5 min at 5EC for 7 days (Mitsuda et al., 1990)

Kind of gas and mixing ratio	**Number of bacteria (survival %)**			
	just after exposure		**after storage**	
Control	1.4 x 10^3	100%	40 x 10^3	100%
Ozone gas	0.9	64	8.0	20
CO_2 gas	1.3	93	30	75
Mixed gas (O_3:CO_2, v/v)				
3:1	0.5	36	8.0	20
2:1	0.6	43	4.0	10
1:1	0.6	43	5.2	13
1:2	1.0	71	6.4	16
1:3	1.2	86	7.1	18

After storing the samples in the same gases in bags at 5EC for 7 days, the number of bacteria increased, although the increases varied with the kind of gas and the mixing ratio of ozone and

carbon dioxide. Not only pure carbon dioxide gas but also pure ozone gas did not reduce the growth of bacteria. However, the retarding effect of the gas mixture was observed. The most effective mixing ratios of ozone and carbon dioxide gas were 2:1, 1:1, 1:2, and 1:3.

Sterilization of Bacterial Cells in an Agar Plate: To find the mechanism of this synergistic effect, a sterilization test for bacterial cells in an agar plate with ozone gas, carbon dioxide gas and a mixture of these gases was carried out. In the usual way, the numbers of the surviving bacterial cells were measured after ozone was bubbled through the bacterial suspension. In this study, however, the sterilization test was carried out on the bacterial cells located in the agar plate.

As shown in Table 2.2.04, the sterilization effect after 5 minutes was restricted, because the bacterial cells that were located on the surface of the agar plate were included as a part of the cells on the plate. Both in the five minutes of sterilization and the storage of the agar plate, the sterilization effect of some samples under the gas mixture was larger than that of samples under ozone and carbon dioxide gas in the single use. Desirable results were obtained when the mixing ratio of the ozone and carbon dioxide was 1:3 and 1:1.

Table 2.2.04. Survival of *Escherichia coli* in different gases and gas mixtures just after exposure at room temperature for 5 min and at 37EC for 48 hours (Mitsuda et al., 1990)

Kind of gas and mixing ratio	**Number of bacteria (survival %)**			
	just after exposure		**after storage**	
Control	440	100%	422	100%
Ozone gas	32	7	86	20
CO_2 gas	163	37	311	74
Mixed gas				
(O_3:CO_2, v/v)	56	13	125	30
3:1	55	13	122	29
2:1	300	68	0	0
1:1	310	70	124	29
1:2	5	1	166	39
1:3				

In addition, the number of bacteria after exposure to carbon dioxide was greater than that for ozone, but the size of the colonies of bacteria was smaller for carbon dioxide. Furthermore, the colonies that resulted after exposure to ozone gas were found inside the samples.

After the investigation on the mechanism of the interaction between carbon dioxide gas and protein, carbon dioxide binding sites in protein in the gas-solid phase system were revealed to be ,-amino, ∀-amino and guanidiurn groups. On the findings of these characteristics of carbon dioxide, the reasons for this synergistic effect observed in this study were considered as follows:

1. Ozone was reported to be bactericidal rather than bacteriostatic and carbon dioxide gas was known to be bacteriostatic. The synergistic effect could not be assumed to be caused by the

simple summation of the bactericidal effect of ozone gas, and the bacteriostatic effect of carbon dioxide gas, because the optimal conditions for the synergistic effect were obtained in a restricted ratio of both gases.

2. The bactericidal effect of ozone gas was assumed to be retained during these storage periods by the quenching effect of carbon dioxide gas to the chain reaction of ozone degradation. Decrease of the bacterial number for the storage period was assumed to be caused by the high solubility and the bacteriostatic effect of carbon dioxide gas to the bacterial cells which were located not only on the surface but also inside the agar plate.

2.2.4 Gorman et al. (1995b) – Beef Brisket Fat

Abstract: Various chemical solutions (5% hydrogen peroxide, 0.5% ozone, 12% trisodium phosphate, 2% acetic acid, and 0.3% commercial sanitizer), water (16 to 74EC spray-washing interventions, and hand-trimming/spray-washing treatments were compared for their ability to remove fecal material and to reduce bacterial contamination on beef brisket fat samples in a model spray-washing cabinet. The samples were inoculated with 2.5 cm^2 of a bovine fecal paste inoculated with *Escherichia coli* (ATCC 11370). Hand-trimming followed by spray-washing with plain water (16 to 74EC when it came in contact with the sample; 20.68 bar pressure; for 36 or 12 s corresponding to chain speeds of 100 or 300 carcasses per h) lowered ($P < 0.05$) microbiological counts, compared to the inoculated control, by 1.41 to 2.50 log colony-forming units (CFU)/cm^2. Additionally, spraying with chemical solutions (16EC; 1.38 bar, 12 or 36 s), before or after spray-washing with plain water (20.68 bar) of 16EC (36 s), 35EC (12 s) or 74EC (12 s) reduced bacterial counts by 1.34 to 2.87, 1.18 to 2.86, or 0.96 to 3.42 log CFU/cm^2 , respectively. Reduction in counts was influenced by water temperature (16 to 74EC, type of chemical solution, and sequence of spray application. **Under the conditions of this study, hydrogen peroxide and ozonated water were more effective ($P < 0.05$) than trisodium phosphate, acetic acid, and a commercial sanitizer when applied after first washing with plain water.** Trisodium phosphate maintained its activity when used before washing with water. In general, water of 74EC caused reductions ($P < 0.05$) exceeding 3.0 log CFU/cm^2, which were higher than those achieved by trimming and spray washing. No spreading of bacteria in areas immediately adjacent to the inoculation site was detected following spray washing.

Materials and Methods

Inoculum Preparation: A pure culture of a streptomycin-resistant *Escherichia coli* strain (ATCC 11370) was prepared at a certified laboratory by incubation for 24 h at 37EC in nutrient broth containing 625 Φg of dihydrostreptomycin/mL. A portion (100 mL) of the culture, diluted to 10^8 colony-forming units (CFU)/mL, was mixed aseptically with 300 g of fresh bovine feces (collected each day of experimentation from the holding pens of a large commercial beef slaughter facility) in a sterile stomacher bag by hand massaging for two min. To obtain the desired paste consistency, sterile water was added to the inoculated fecal material to achieve a ratio of 3 parts added liquid to 1 part fecal material. The inoculum then was transported from the laboratory in coolers with ice packs to a commercial slaughter facility (within a distance of one mile) for inoculation of samples on the same day.

Sample Handling and Decontaminating Treatments: Hot (< 15 min postmortem) adipose tissue pieces, cut from the brisket area of beef carcasses prior to any routine carcass washing or trimming, were transported to a room adjacent to the slaughter area using clean plastic trays. Before testing, each piece was cut aseptically with a sterile knife blade into a 10 by 10 cm square portion, which was inoculated with a plastic inoculating loop used to transfer the inoculated fecal paste to its center. The total inoculation area was 2.5 cm^2 and was obtained by using a 0.625-cm^2 inoculating loop and transferring inoculum to the adipose tissue for four times. Each analytical sample unit consisted of three (10 by 10 cm) pieces of adipose tissue (from three beef carcasses) which, after inoculation, were held for 15 min at room temperature (20EC) to allow for attachment of fecal material and bacteria. The three pieces of adipose tissue then were treated by hand-trimming (using a sterile knife blade and forceps to remove all visible fecal contamination) or by spray-washing under specified conditions.

Spray washing was done in a model, conveyorized, two-chamber, spray-wash cabinet, especially designed and built for these studies. The first washing chamber contained one 0.3125-cm (MEG 2150) oscillating nozzle, with oscillation set at 80 rpm to cover the entire length of the sample being washed. Only water was administered in this chamber, and the variables studied included slaughter chain speed (100 or 300 carcasses per h, corresponding to 36 or 12 s of exposure time), spray-washing pressure (2.76, 6.89, or 20.68 bar), and water temperature (16, 35, 66, or 74 ∀ 5EC). In the second chamber the sanitizing agents were applied at 16EC, 1.38 bar, and for 12 or 36 s. The hot water and chemical solutions were applied in two sequences, one following and the second preceding the plain water spray-washing treatment.

Visual Evaluation and Sampling: The treatment samples were evaluated visually by 2 or 3 people for any remaining fecal material. Visual scores were based on a 5-point scale in which 0 indicated no visible fecal material contamination, 1 indicated sparse evidence of fecal material, 3 indicated presence of fecal material, and 5 indicated obvious, dense fecal material contamination (detected on the unwashed inoculated samples). The samples were also evaluated visually before and after hand-trimming and/or spray-washing.

Following hand-trimming and/or spray-washing or before any treatment for the controls -- as appropriate -- tissue samples were taken, aseptically, from each piece of adipose tissue, using sterile cork borers (3.175-cm diameter), and a sterile scalpel and forceps. The samples were taken from the center (at the site of inoculation) of all three pieces of adipose tissue for each sampling unit and placed (all three samples) in a single sterile stomacher bag for subsequent microbiological analysis; this was designated as sample A. Two additional similar samples were taken, aseptically, from positions above and below the center of the brisket adipose tissue pieces and placed into another single sterile stomacher bag for subsequent microbial analysis, this was designated as sample B and consisted of six individual pieces of fat. The B sample was taken to determine the effect of spray-washing in causing translocation of microorganisms (from the center site to surrounding sites) through splashing or run-off and contamination of areas adjacent to the inoculation site. The A and B samples then were placed in coolers with ice packs for transportation to the laboratory (within a distance of one mile) for subsequent microbiological analysis; all testing was initiated within 2 h of hand-trimming or spray-washing.

Microbiological Analyses: Samples were diluted to a 10-1 dilution with sterile phosphate buffer (pH 7.0, KH_2PO_4) and then stomached using a model 400 stomacher for 2 min. Plating

was done on nutrient agar without or with 625 Φg dihydrostreptomycin/mL with a spiral plating system. The inoculated plates were incubated at 37EC for 48 h, then colonies were counted using a model 800 processor with a model 500 laser colony counter. The results were expressed and recorded in CFU/cm^2 of surface adipose tissue.

Statistical Analysis: A one-way completely randomized design was used in the study, which involved six replicates for each treatment, and least-square means (LSM) were calculated from the six replications. The LSM were separated using the least significant difference (LSD) procedure with the alpha level set at 0.05.

Results and Discussion

Table 2.2.05 and 2.2.06 summarize visual evaluation scores for fecal material contamination and microbiological counts for the site of inoculation (A) for the various treatment methods employed, including ozone (OZ 0.5% in water). The uninoculated samples contained 5.42 and 4.28 log CFU/cm^2 of total plate and streptomycin-resistant counts (data not shown), respectively, and these counts increased, after application of the inoculated fecal paste, to 6.66 and 6.26 log CFU/cm^2, respectively. The microbiological counts for the areas adjacent to the site of inoculation (B) are presented in Tables 2.2.07 and 2.2.08. These counts were 4.89 and 4.23 log CFU/cm^2 for total plate and streptomycin-resistant counts, respectively, in the uninoculated samples (data not shown), and 4.92 and 4.43 log CFU/cm^2 , respectively, in the inoculated samples. Because trends in the changes of microbiological counts after application of the spray-washing or hand-trimming treatments were similar between total plate and streptomycin-resistant bacterial counts, only the total plate counts are discussed in the remainder of this paper.

Reduction in Microbiological Counts: Trimming alone or trimming followed by a single spray-washing treatment of plain water (16 to 74EC; 20.68 bar; 12 or 36 s) significantly ($P < 0.05$) reduced the microbiological counts compared to the inoculated control. This indicates that the required trimming under the zero tolerance directive of FSIS-USDA reduces microbiological contamination after carcasses are contaminated with fecal material. A significant amount of contamination remained on the samples, however, even after trimming or trimming/spray-washing treatments. This study included evaluation of water temperatures (16, 35, 66, or 74EC), spraying pressures (2.76, 6.89, or 20.68 bar) and chain speeds (12 or 36 s), in an attempt to provide information that could be useful to meat packing plants operating under different conditions. Approximate reductions in bacterial counts achieved by a single spray-washing (no trimming) treatment of plain water were in the range of 1 to 2 log CFU/cm^2 compared to the inoculated/untrimmed/unwashed control. The higher pressures (i.e., 20.68 bar) generally were more effective. It is interesting to note that these washing (no trimming) treatments were as effective ($P > 0.05$) (at the higher pressure) as the trimming and washing combinations discussed earlier.

A large number of treatments in this study involved use of the two spray-washing chambers of the cabinet: one operating with plain water and the second with cold (16EC water alone or in combination with chemical interventions (including ozone). The plain water treatment was examined at 16EC, 20.68 bar, and 36 s, as well as at 35EC or 74EC, 20.68 bar, and 12 s (Tables 2.2.05 and 2.2.06). The chemical interventions were applied in the second chamber as solutions in 16EC water at 1.38 bar and for 12 s (300 carcasses per h). The total experiment was

performed in two sequences; in the first sequence, the plain water treatments were followed by the chemical interventions (Table 2.2.05), and in the second sequence, the chemical interventions were followed by the plain water treatments (Table 2.2.06).

Table 2.2.05. Visual fecal contamination scores and microbiological counts of beef brisket adipose tissue samples artificially inoculated and hand-trimmed or spray-washed under specific treatment conditions (sample location A, inoculation site) (Gorman et al., 1995b)

Treatments							
	First Wash			Microbiological counts		Visual evaluation[a]	
	Solution temperature (°C)	Pressure (bar)	Exposure time (s)	Total plate [log CFU/cm² (SD)]	Streptomycin-resistant [log CFU/cm² (SD)]	Before treatment [score (SD)]	After treatment [score (SD)]
Inoculated/Untrimmed/Unwashed				6.66 (0.35)^{a}	6.26 (0.58)^{a}	5.00 (0.00)	5.00 (0.00)^{a}
Inoculated/Trimmed/Unwashed				4.16 (1.16)^{ef}	4.24 (1.04)^{defg}	5.00 (0.00)	0.35 (0.41)^{hi}
Inoculated/Trimmed/Washed (16°C water, 20.68, 36 s)				5.05 (0.59)^{bcd}	4.35 (0.37)^{cdefg}	5.00 (0.00)	0.10 (0.22)^{hi}
Inoculated/Trimmed/Washed (35°C water, 20.68, 12 s)				4.34 (0.89)^{def}	3.97 (0.67)^{fg}	5.00 (0.00)	1.00 (1.83)^{b}
Inoculated/Trimmed/Washed (66°C water, 20.68, 12 s)				4.78 (0.51)^{cde}	4.08 (0.33)^{efg}	5.00 (0.00)	0.33 (0.41)^{hi}
Inoculated/Trimmed/Washed (74°C water, 20.68, 12 s)				5.22 (0.22)^{bc}	4.85 (0.34)^{bcd}	5.00 (0.00)	0.14 (0.24)^{hi}
Water	16	20.68	36	4.60 (0.44)^{cde}	3.87 (0.18)^{g}	5.00 (0.00)	0.25 (0.27)^{hi}
Water	35	2.76	12	5.17 (0.26)^{bc}	4.72 (0.54)^{bcde}	5.00 (0.00)	0.83 (0.41)^{hi}
Water	35	6.89	12	4.66 (0.51)^{cde}	4.01 (0.82)^{efg}	5.00 (0.00)	0.83 (0.26)^{hi}
Water	35	20.68	12	5.19 (0.16)^{bc}	4.63 (0.63)^{bcdef}	5.00 (0.00)	0.42 (0.49)^{hi}
Water	66	2.76	12	5.81 (0.24)^{b}	5.09 (0.63)^{bc}	5.00 (0.00)	0.17 (0.41)^{hi}
Water	66	6.89	12	5.19 (1.33)^{bc}	4.94 (1.07)^{bc}	5.00 (0.00)	0.00 (0.00)^{i}
Water	74	2.76	12	5.62 (0.21)^{b}	5.32 (0.20)^{b}	5.00 (0.00)	0.00 (0.00)^{i}
Water	74	6.89	12	5.68 (0.10)^{b}	5.16 (0.53)^{b}	5.00 (0.00)	0.00 (0.00)^{i}
Water	74	20.68	12	4.74 (0.79)^{cd}	4.05 (0.41)^{efg}	5.00 (0.00)	0.17 (0.26)^{hi}

[a-g] Means in the same column with different superscript letters are significantly different ($P < 0.05$).

[a] Score of 0, no visible fecal material contamination; score of 1, sparse evidence of fecal material; score of 3, presence of fecal material; score of 5, obvious, dense fecal material contamination.

When the first spray-washing action involved use of plain water at 16, 35, or 74EC followed by additional plain water at 16EC, the reductions in total plate counts were 1.35, 1.24, and 3.37 log CFU/cm^2, respectively (Table 2.2.05). ***When the first spray-washing treatments of 16 or 35EC plain water were followed by chemical solution interventions, the most effective sanitizing agents were hydrogen peroxide (5%) and ozonated water (0.5%), achieving reductions in total plate counts of 2.60 to 2.87 and 2.72 to 2.86 log CFU/cm^2, respectively.*** The least effective chemical agents were the commercial sanitizer (0.3%) and acetic acid (2%), achieving reductions of 1.43 to 1.94 and 2.01 to 2.02 log CFU/cm^2, respectively, while trisodium phosphate (12%) reduced the contamination by 2.26 to 2.30 log CFU/cm^2. When the first spray-washing treatment involved use of 74EC plain water, reductions in total plate counts achieved by any of the treatments that followed in the second chamber of the spray-washing cabinet, including the 16EC plain water, were 3.06 to 3.42 log CFU/cm^2 (Table 2.2.05). Thus, when water of 74EC was employed, reductions in contamination exceeded 3.0 log CFU/cm^2, irrespective of the presence or absence of chemical sanitizers. ***When the spray-washing water temperature was 16 or 35EC, a subsequent treatment with chemical solutions increased reductions in total plate counts on beef brisket fat, with hydrogen peroxide and ozonated water being the most effective treatments.***

Table 2.2.06. Visual fecal contamination scores and microbiological counts of beef brisket adipose samples artificially inoculated and hand trimmed or sanitized then spray-washed first under specific treatment conditions (sample location A, inoculation site) (Gorman et al., 1995b)

Treatments								Microbiological counts		Visual evaluation[x]	
First wash				Second wash							
	Solution temp. (°C)	Pressure (bar)	Exposure time (s)		Solution temp. (°C)	Pressure (bar)	Exposure time (s)	Total plate [log CFU/cm² (SD)]	Streptomycin-resistant [log CFU/cm² (SD)]	Before treatment [score (SD)]	After treatment [score (SD)]
Inoculated/Untrimmed/Unwashed								6.66 (0.35)[a]	6.26 (0.58)[a]	5.00 (0.00)	5.00 (0.00)[a]
Inoculated/Trimmed/Unwashed								4.16 (1.16)[cdefgh]	4.24 (1.04)[fghij]	5.00 (0.00)	0.35 (0.41)[hi]
Inoculated/Trimmed/Washed (16°C water, 20.68, 100 carcasses/h)								5.05 (0.59)[bcd]	4.35 (0.37)[defgh]	5.00 (0.00)	0.10 (0.22)[i]
Inoculated/Trimmed/Washed (35°C water, 20.68, 300 carcasses/h)								4.34 (0.89)[defg]	3.97 (0.67)[ghijk]	5.00 (0.00)	1.00 (1.83)[b]
Inoculated/Trimmed/Washed (66°C water, 20.68, 300 carcasses/h)								4.78 (0.51)[bcdef]	4.08 (0.33)[fghij]	5.00 (0.00)	0.33 (0.41)[hi]
Inoculated/Trimmed/Washed (74°C water, 20.68, 300 carcasses/h)								5.22 (0.22)[bc]	4.85 (0.34)[bcde]	5.00 (0.00)	0.14 (0.24)[i]
Water	16	20.68	36	Water	16	1.38	36	5.31 (0.87)[bc]	4.98 (0.70)[bcd]	5.00 (0.00)	0.00 (0.00)[i]
Water	16	20.68	36	TSP[m] (12%)	16	1.38	36	4.36 (0.53)[defg]	3.72 (0.44)[hijkl]	5.00 (0.00)	0.25 (0.42)[hi]
Water	16	20.68	36	AA (2%)	16	1.38	36	4.64 (0.43)[cdefg]	4.40 (0.45)[defg]	5.00 (0.00)	0.50 (0.45)[hi]
Water	16	20.68	36	HP (5%)	16	1.38	36	3.79 (0.85)[ghij]	3.87 (0.62)[ghijk]	5.00 (0.00)	0.17 (0.41)[i]
Water	16	20.68	36	OZ (0.5%)	16	1.38	36	3.94 (0.70)[fghij]	3.74 (0.42)[hijkl]	5.00 (0.00)	0.17 (0.26)[i]
Water	16	20.68	36	CS (0.3%)	16	1.38	36	4.72 (0.71)[bcde]	4.58 (0.51)[cdefg]	5.00 (0.00)	0.25 (0.42)[hi]
Water	35	20.68	12	Water	16	1.38	12	5.42 (0.93)[b]	5.38 (0.27)[b]	5.00 (0.00)	0.00 (0.00)[i]
Water	35	20.68	12	TSP (12%)	16	1.38	12	4.40(1.03)[defg]	3.44(0.33)[kl]	5.00 (0.00)	0.17(0.26)[i]
Water	35	20.68	12	AA (2%)	16	1.38	12	4.65 (0.42)[cdefg]	4.34 (0.57)[defgh]	5.00 (0.00)	1.08 (0.58)[b]
Water	35	20.68	12	HP (5%)	16	1.38	12	4.06 (0.49)[defghi]	4.19 (0.94)[efghij]	5.00 (0.00)	0.00 (0.00)[i]
Water	35	20.68	12	OZ (0.5%)	16	1.38	12	3.80 (0.49)[ghij]	3.70 (0.54)[ijkl]	5.00 (0.00)	0.08 (0.20)[i]
Water	35	20.68	12	CS (0.3%)	16	1.38	12	5.23 (0.74)[bc]	5.03 (0.58)[bc]	5.00 (0.00)	0.08 (0.20)[i]
Water	74	20.68	12	Water	16	1.38	12	3.29 (0.12)[j]	3.24 (0.00)[l]	5.00 (0.00)	0.08 (0.20)[i]
Water	74	20.68	12	TSP (12%)	16	1.38	12	3.60 (0.34)[hij]	3.48 (0.38)[jkl]	5.00 (0.00)	0.25 (0.42)[hi]
Water	74	20.68	12	AA (2%)	16	1.38	12	3.24 (0.00)[j]	3.24 (0.00)[l]	5.00 (0.00)	0.25 (0.42)[hi]
Water	74	20.68	12	HP (5%)	16	1.38	12	3.44 (0.36)[ij]	3.24 (0.00)[l]	5.00 (0.00)	0.08 (0.20)[i]
Water	74	20.68	12	OZ (0.5%)	16	1.38	12	3.49 (0.32)[ij]	3.24 (0.00)[l]	5.00 (0.00)	0.17 (0.26)[i]
Water	74	20.68	12	CS (0.3%)	16	1.38	12	3.29 (0.12)[j]	3.24 (0.00)[l]	5.00 (0.00)	0.17 (0.26)[i]

[x] Score of 0, no visible fecal material contamination; score of 1, sparse evidence of fecal material; score of 3, presence of fecal material; score of 5, obvious, dense fecal material contamination.

[a-l] Means in the same column with different superscript letters are significantly different ($P < 0.05$).

[m] Abbreviations: SD, standard deviation; TSP, trisodium phosphate; AA, acetic acid; HP, hydrogen peroxid; OZ, ozonated water; CS, commercial sanitizer.

When the sequence of the application of the interventions was reversed (application of the sanitizing agents followed by plain water spray-washing), the effectiveness of the sanitizers was different, with trisodium phosphate (12%) being the most effective treatment (Table 2.2.06). When the chemical intervention preceded the water treatments of 16 or 35EC, reductions in levels of total plate counts achieved by water alone, trisodium phosphate (12%), acetic acid (2%), ozonated water (0.5%), hydrogen peroxide (5%), and the commercial sanitizer (0.3%) were 2.13 to 2.80, 2.30 to 2.33, 1.33 to 2.14, 1.36 to 1.40, 1.18 to 1.34, and 1.21 to 1.42 log CFU/cm^2, respectively. When the spray-washing treatment that followed application of the chemical sanitizer involved water at 74EC, the only agent that maintained its activity and caused reductions in total plate counts exceeding 3 log CFU/cm^2 was trisodium phosphate (12%). The loss of activity of chemicals when followed by plain-water spray-washing possibly was due to physical removal or dilution of the sanitizing agents, while trisodium phosphate would appear to act by a different mechanism in decontaminating beef adipose tissue. Thus, the sequence of application would be important if chemical interventions were to be used, but chemical interventions would not be necessary if hot water (74EC) was the intervention chosen for carcass decontamination.

In addition, the variation in counts among samples spray-washed with 74EC water, as measured by the standard deviations shown in Tables 2.2.05 to 2.2.06 generally was lower than that of hand-trimming and spray-washing treatments at lower temperatures. Furthermore, hot-water spray washing resulted in greater reductions in bacterial counts than combinations of hand trimming and washing. Thus, these data indicate that spray washing with hot water is an effective means of reducing microbial counts on the external fat surfaces of beef. The reduction in total bacterial counts by use of hot water (74EC), which exceeded 3.00 log CFU/cm^2, compared favorably with the reduction of 1.44 to 2.32 log CFU/cm^2 achieved by the presently used treatments of hand-trimming followed by spray-washing (Tables 2.2.05 to 2.2.06). ***The data also indicated that chemical treatments such as hydrogen peroxide and ozonated water may be worth further testing for their potential application in packing-plant operations that are unable to supply hot water.***

Effect of Spray-Washing on Spreading of Contamination: Bacterial numbers in the areas of beef brisket samples immediately adjacent to the inoculation site were always lower than counts on the inoculation site, both before and after application of spray-washing or chemical interventions (Tables 2.2.05 to 2.2.08). The results demonstrate that there was no spreading of the bacteria in the inoculum onto areas immediately adjacent to the inoculation site through the spray-washing action, or that the spray-washing treatments diluted bacterial contamination, resulting in reduced counts. Comparing the counts from the areas adjacent to the inoculation site (location B), which were recovered after application of specific treatments, with the original control counts, the counts were lower ($P < 0.05$) for those samples washed with 74EC water (Tables 2.2.07 to 2.2.08). Thus, spray-washing under the conditions of this study either diluted the contamination or did not move or spread the bacteria to areas adjacent to the site of artificial contamination (location A). Use of spray-washing pressures of less than 20.7 bar should not have resulted in bacteria being embedded into the tissue.

Reductions in Visual Fecal Evaluation Scores: All treatments achieved virtual elimination of the visible fecal material contaminants that had been placed on the samples before processing (Tables 2.2.05 to 2.2.06). The change in scores from before treatment (score of 5) to after treatment was significantly ($P < 0.05$) lower and in many instances the decrease was between 4.17 and 5.00, suggesting that these treatments produced very clean sample surfaces (from a physical contaminant standpoint). Therefore, the treatments tested not only reduced bacterial counts, but also cleaned the surfaces of the fat.

Table 2.2.07. Microbiological counts of beef brisket adipose tissue samples obtained at locations (B) adjacent to the inoculation site before and after hand-trimming or sanitizing and spray-washing under specific treatment conditions (Gorman et al., 1995b)

Treatments								Microbiological counts	
First wash				Second wash					
	Solution temperature (°C)	Pressure (bar)	Exposure time (s)		Solution temperature (°C)	Pressure (bar)	Exposure time (s)	Total plate [log CFU/cm² (SD)]	Streptomycin-resistant [log CFU/cm² (SD)]
Inoculated/Untrimmed/Unwashed								4.92 (0.94)[ab]	4.43 (0.93)[bcd]
Inoculated/Trimmed/Unwashed								4.90 (0.76)[ab]	3.80 (0.79)[efg]
Inoculated/Trimmed/Washed (16°C water, 20.68, 36 s)								4.56 (0.58)[bcd]	3.90 (0.55)[cdefg]
Inoculated/Trimmed/Washed (35°C water, 20.68, 12 s)								3.85 (0.85)[def]	3.66 (0.82)[efgh]
Inoculated/Trimmed/Washed (66°C water, 20.68, 12 s)								4.89 (0.35)[ab]	3.94 (0.10)[cdefg]
Inoculated/Trimmed/Washed (74°C water, 20.68, 12 s)								4.87 (0.22)[ab]	4.31 (0.71)[bcdef]
Water	16	20.68	36	Water	16	1.38	36	4.93 (1.33)[ab]	5.06 (0.76)[a]
Water	16	20.68	36	TSP[a](12%)	16	1.38	36	4.28 (0.76)[bcdefg]	3.69 (0.86)[efgh]
Water	16	20.68	36	AA (2%)	16	1.38	36	4.47 (0.55)[bcd]	4.25 (0.53)[bcdefg]
Water	16	20.68	36	HP (5%)	16	1.38	36	3.26 (0.28)[fgh]	3.01 (0.20)[hi]
Water	16	20.68	36	OZ (0.5%)	16	1.38	36	3.33 (0.40)[fgh]	2.93 (0.00)[i]
Water	16	20.68	36	CS (0.3%)	16	1.38	36	3.95 (0.82)[cdef]	4.61 (0.63)[abc]
Water	35	20.68	12	Water	16	1.38	12	5.53 (0.38)[a]	4.84 (0.86)[ab]
Water	35	20.68	12	TSP (12%)	16	1.38	12	5.00 (0.76)[ab]	4.08 (0.92)[cdefg]
Water	35	20.68	12	AA (2%)	16	1.38	12	4.31 (0.32)[bcd]	3.84 (0.52)[defg]
Water	35	20.68	12	HP (5%)	16	1.38	12	2.93 (0.00)[i]	3.06 (0.32)[hi]
Water	35	20.68	12	OZ (0.5%)	16	1.38	12	3.45 (0.42)[efgh]	2.98 (0.13)[hi]
Water	35	20.68	12	CS (0.3%)	16	1.38	12	4.57 (1.04)[bc]	4.17 (0.87)[bcdefg]
Water	74	20.68	12	Water	16	1.38	12	3.48 (0.44)[efgh]	3.23 (0.48)[ghi]
Water	74	20.68	12	TSP (12%)	16	1.38	12	3.76 (0.89)[defg]	3.57 (1.06)[efghi]
Water	74	20.68	12	AA (2%)	16	1.38	12	2.93 (0.00)[i]	3.05 (0.29)[hi]
Water	74	20.68	12	HP (5%)	16	1.38	12	3.11 (0.28)[gh]	2.93 (0.00)[i]
Water	74	20.68	12	OZ (0.5%)	16	1.38	12	3.51 (0.50)[defgh]	2.93 (0.00)[i]
Water	74	20.68	12	CS (0.3%)	16	1.38	12	2.98 (0.13)[i]	2.93 (0.00)[i]

[a-i] Means in the same column with different superscript letters are significantly different ($P < 0.05$).
[a] TSP, trisodium phosphate; AA, acetic acid; HP, hydrogen peroxide; OZ, ozonated water; CS, commercial sanitizer.

Conclusions

The results of this study indicated that application of hot water (74EC at the surface of the sample) in beef spray-washing processes resulted in reductions of microbiological counts of the order of 3.0 log CFU/cm^2 compared to the combination of hand-trimming and spray-washing with colder (< 35EQ water, which achieved reductions in total plate counts of 1.44 to 2.32 log CFU/cm^2. Furthermore, spray-washing with hot water resulted in less variability in bacterial counts obtained after treatment compared to hand-trimming and/or spray-washing with water of lower temperatures. This greater variability in bacterial counts for hand-trimming treatments indicated the potential for cross-contamination during the process.

Table 2.2.08. Microbiological counts of beef brisket adipose tissue samples obtained at locations (B) adjacent to the inoculation site before and after hand-trimming or sanitizing then spray-washing under specific treatment conditions (Gorman et al., 1995b)

Treatments									
First wash				Second wash				Microbiological counts	
	Solution temperature (°C)	Pressure (bar)	Exposure time (s)		Solution temperature (°C)	Pressure (bar)	Exposure time (s)	Total plate [log CFU/cm² (SD)]	Streptomycin-resistant [log CFU/cm² (SD)]
Inoculated/Untrimmed/Unwashed								4.92 (0.94)^{abc}	4.43 (0.93)^{bcd}
Inoculated/Trimmed/Unwashed								4.90 (0.76)^{abc}	3.80 (0.79)^{efgh}
Inoculated/Trimmed/Washed (16°C water, 20.68, 36 s)								4.56 (0.58)^{bcd}	3.90 (0.55)^{defgh}
Inoculated/Trimmed/Washed (35°C water, 20.68, 12 s)								3.85 (0.85)^{efg}	3.66 (0.82)^{fgh}
Inoculated/Trimmed/Washed (66°C water, 20.68, 12 s)								4.89 (0.35)^{abc}	3.94 (0.10)^{defgh}
Inoculated/Trimmed/Washed (74°C water, 20.68, 12 s)								4.87 (0.22)^{abc}	4.31 (0.71)^{bcdef}
Water	16	1.38	36	Water	16	20.68	36	3.72 (0.49)^{cdefg}	3.44 (0.68)^{efgh}
TSP[a] (12%)	16	1.38	36	Water	16	20.68	36	3.24 (0.30)^{gh}	2.93 (0.00)^{i}
AA (2%)	16	1.38	36	Water	16	20.68	36	4.14 (0.29)^{abcde}	4.17 (0.31)^{cdefg}
HP (5%)	16	1.38	36	Water	16	20.68	36	5.20 (0.27)^{ab}	4.86 (0.18)^{ab}
OZ (0.5%)	16	1.38	36	Water	16	20.68	36	4.83 (0.27)^{abc}	4.69 (0.41)^{abc}
CS (0.3%)	16	1.38	36	Water	16	20.68	36	4.96 (0.43)^{abc}	4.65 (0.87)^{abc}
Water	16	1.38	12	Water	35	20.68	12	3.76 (0.75)^{defg}	3.41 (0.55)^{efgh}
TSP (12%)	16	1.38	12	Water	35	20.68	12	3.63 (0.46)^{defg}	2.93 (0.00)^{i}
AA (2%)	16	1.38	12	Water	35	20.68	12	5.07 (0.28)^{abc}	5.06 (0.31)^{a}
HP (5%)	16	1.38	12	Water	35	20.68	12	4.97 (0.40)^{abc}	4.75 (0.39)^{abc}
OZ (0.5%)	16	1.38	12	Water	35	20.68	12	4.52 (0.82)^{cdef}	4.10 (0.35)^{abcdef}
CS (0.3%)	16	1.38	12	Water	35	20.68	12	5.21 (0.12)^{ab}	5.00 (0.17)^{a}
Water	16	1.38	12	Water	74	20.68	12	3.41 (0.53)^{fgh}	3.30 (0.49)^{gh}
TSP (12%)	16	1.38	12	Water	74	20.68	12	2.93 (0.00)^{h}	2.93 (0.00)^{i}
AA (2%)	16	1.38	12	Water	74	20.68	12	4.93 (0.29)^{abc}	4.83 (0.24)^{ab}
HP (5%)	16	1.38	12	Water	74	20.68	12	5.01 (0.08)^{abc}	4.42 (0.34)^{bcd}
OZ (0.5%)	16	1.38	12	Water	74	20.68	12	4.64 (0.40)^{bcd}	3.97 (0.61)^{defg}
CS (0.3%)	16	1.38	12	Water	74	20.68	12	5.33 (0.50)^{a}	4.11 (0.30)^{cdef}

[a–i] Means in the same column with different superscript letters are significantly different ($P < 0.05$).
[a] TSP, trisodium phosphate; AA, acetic acid; HP, hydrogen peroxide; OZ, ozonated water; CS, commercial sanitizer.

Chemical interventions in the form of spray-washing solutions, such as hydrogen peroxide, ozonated water, and trisodium phosphate, increased reduction of microbial counts when following spraying with water of lower temperatures (e.g., 35EC, but their activity was overshadowed when their application followed spraying with hot (74EC water. The sequence of application for the chemical interventions was important since hydrogen peroxide and ozonated water lost their activity when their application preceded spraying with plain water, while trisodium phosphate maintained the same activity both when preceded or followed by plain water spray-washing. This finding suggests differences in mechanisms of decontamination among the interventions tested. Spray-washing treatments caused no spreading of the bacterial inoculum onto areas immediately adjacent to the inoculation site or diluted the contamination resulting in lower counts after spray washing. Trimming and spray-washing treatments achieved extensive removal of visible fecal material contaminants from the samples. Based on the conditions and results of this study, the use of elevated temperature (e.g., 74EC water may be the most useful treatment in the decontamination and the removal of fecal material from beef carcasses. As an alternative to hot water, the chemical interventions should be investigated for their potential application with water sprays of lower temperature (16 to 35EC).

2.2.5 Reagan et al. (1996) – Beef Carcasses

Abstract: A study to compare procedures and interventions for removing physical and bacterial contamination from beef carcasses was conducted in six carcass conversion operations that were representative of modern, high-volume plants and located in five different states. Treatment procedures included trimming, washing, and the current industry practice of trimming followed by washing. In addition, hot (74 to 87.8EC at the pipe) water washing and rinsing with ozone (0.3 to 2.3 ppm) or hydrogen peroxide (5%) were applied as intervention treatments. Beef carcasses were deliberately contaminated with bovine fecal material at >4.0 log colony-forming units (CFU)/cm^2 in order to be better able to observe the decontaminating effects of the treatments. Samples (10 by 15 cm, 0.3 to 0.5 cm thick) for microbiological testing were excised as controls or after application of each procedure or intervention and analyzed for aerobic mesophilic plate counts, *Escherichia coli* Biotype I counts, and presence or absence of *Listeria spp*., *Salmonella spp*., and *Escherichia coli* O157:117. Average reductions in aerobic plate counts were 1.85 and 2.00 log CFU/cm^2 for the treatments of trimming-washing and hot water washing, respectively. ***Hydrogen peroxide and ozone reduced aerobic plate counts by 1.14 and 1.30 log CFU/cm^2, respectively.*** In general, trimming and washing of beef carcasses consistently resulted in low bacterial populations and scores for visible contamination. However, the data also indicated that hot- (74 to 87.8EC at the pipe) water washing was an effective intervention that reduced bacterial and fecal contamination in a consistent manner.

Materials and Methods

Selection of Beef Carcasses: Six beef-slaughtering operations were selected as being representative of modern, high-volume plants. The plants were geographically dispersed, being located in five states, and were operated by four different companies. Four of the plants processed predominantly fed steers and heifers, while the other two processed mostly nonfed cows. The carcasses used for testing were randomly selected at 5- to 10-min intervals from the carcasses being processed (from 100 to 400 head per h, depending on the particular plant), and deliberately contaminated with fecal material obtained from the external surface of the hide of each carcass. The carcasses were contaminated by manual digitation on the inside round at the "high-rimmer" area of processing, immediately after the hide was opened, to create an area of contamination approximately 1.9 cm in diameter (ca. 2.84 cm^2). Testing was performed during the months of June and July 1994, and it was spread over 3 days in each plant with 8 carcasses of each treatment tested on a given day.

Intervention Treatments: Four primary treatments were evaluated in each of the six packing plants: inoculated, not treated control (CNT); trimmed only (T); washed only (W); and the combination of trimmed and washed (TW). The control (CNT) carcasses were neither trimmed nor washed in the area of contamination (before sampling), while the trimmed and washed (TW) carcasses were subjected to the current industry practice of trimming to remove all visible contamination and then washing using automated spray-washers before entrance of the carcasses into the chiller. Carcasses were identified with tags for specific treatments and the areas to be trimmed and sampled were circled with purple, edible ink. Carcasses were subjected to standard trimming practices, but were sampled prior to final washing. Knife-trimming (to remove visible contamination) was performed by plant personnel to meet USDA-FSIS zero-tolerance standards for removing fecal and other visible material The plant personnel trimming carcasses were

instructed to routinely immerse the knife (approximately 15 cm in length) and the hook in hot (82EC) water prior to touching a new carcass surface. Trimming varied among individuals, but generally involved placing the hook above the contaminated area and, in one motion downward, removing the contaminated portion.

The W carcasses were not trimmed in the marked area of contamination, but they were processed through the standard automated spray-washer before sampling. The approximate length of the cabinets was 4 and 11 m for two of the Cary cabinets, while the length of the spray was approximately 3 and 7 m, respectively. In all of the washers, the angle of spray was 25E, but three also had bars at 0E. All washers had four type #2510 nozzles, while three also had bar-type four-hole nozzles. The total water output ranged from a low of 605 to a high of 2,683 liters/min. The water temperatures during normal washing ranged between 28 and 42EC; the pressures between 410 and 2,758 kPa and the spray-washing times between 18 and 39 s.

The experimental intervention treatments of a hot- (74 to 87.8EC at the pipe) water final wash with no trimming (HW), no trimming but carcass rinsing with ozonated (0.3 to 2.3 ppm) water (OZ) after final washing, and no trimming but carcass rinsing with hydrogen peroxide (5% solution) after final washing (PER) were evaluated at two of three plants (two fed steer and heifer plants and one nonfed cow plant). Because of differences in facilities and equipment between the three plants, there was some variation in the level and extent of application of these intervention treatments. The specific conditions-where available or accessible-for each intervention in each plant are given in Table 2.2.09.

Sampling: In practice, the CNT and T carcasses were sampled on the final trimming rail of the slaughtering chain before washing, while the W and TW carcasses were sampled immediately after washing. Each sample, consisting of a 10 by 15 cm area (150 cm^2) and approximately 0.3 to 0.5 cm thick, was aseptically excised from the original contaminated area of the inside round using standardized sterile templates. The broad area of the inside round of carcasses to be used in the study was marked with purple edible ink. In addition, test carcasses were appropriately tagged. After excision, the samples were immediately chilled on ice, and shipped with "blue ice" packets in insulated containers by overnight air express to the analytical laboratory (Chicago, IL). The temperature of the samples was determined on arrival at the analytical laboratory, and samples were inspected for any obvious signs of temperature abuse. The samples were considered to have been properly maintained during shipment if they were received by the laboratory within 24 h, the sample temperature was below 5EC, and the "blue ice" packets were still frozen.

Table 2.2.09. Description of hot-water and chemical-intervention treatments evaluated for decontamination of beef carcasses (Reagan et al., 1996)

Treatment	Plant 1	Plant 2	Plant 3
Hot Water (HW)			
Water Temp. EC at pipe	NP [a]	74	87.8
Wash duration (s) [b]		18	11
Pressure (kPa)		2,413	1,310
Ozonated water (OZ)			
Concentration (ppm)	0.3-0.9	2.3	NP
Rinse duration (s)	3	13	NP
Pressure (kPa)	138	138	NP
Hydrogen Peroxide (PER)			
Concentration (%, vol/vol)	5.0	5.0	NP
Rinse duration (s)	3	13	NP
Pressure (kPa)	138	138	NP

[a] Not performed.

[b] The wash cabinet in plant 3 provided a continuous wash of the entire carcass; the cabinet in plant 2 provided for a three-stage, sequential wash, beginning at the hind legs and moving down the carcass. The result of this design is that, although the total wash was 22 seconds, each portion of the carcass received a wash for a period equivalent to approximately 33% of that time.

Microbiological Analyses: Samples were weighed and homogenized or stomached for 2 min in 200 mL of Butterfield's phosphate buffer. A Waring blender was used to homogenize samples from plant 1, while a Stomacher 400 was used to homogenize samples from plants 2 through 6. Homogenates were analyzed for *Salmonella spp*., *Listeria spp*., *Escherichia coli* O157:H7, aerobic plate counts, and *E. coli* counts. Analysis for each of the three pathogens used portions of 25 mL from the stomached samples (20% of the blended samples) and determined presence or absence of the pathogen in a sample. The lactose preenrichment method was used for *Salmonella spp*. A two-step broth enrichment procedure was used for *Listeria spp*., with the second broth being incubated for 40 to 48 h and then streaked for isolation onto modified Oxford medium and lithium chloride phenylethanol moxalactain agar for isolation of the organism. *Escherichia coli* O157:H7 was isolated and identified by the procedure of Okrend et al. Aerobic plate counts and *E. coli* Biotype I most probable numbers were determined according to standard procedures. Colony-forming units per gram were converted to colony-forming units per cm^2 by multiplying the count by the sample weight and dividing by the fascia surface area of the sample. The sensitivities of the pathogen detection methods were 0.05 and 0.03 organisms per cm^2 for stomached and blended samples, respectively.

The lowest numerical visual score (0.16) obtained after application of the decontamination treatments was that for the trimmed and washed (TW) samples (Table 2.2.10). This score was significantly ($P < 0.05$) lower than those obtained with any other treatment, indicating that the currently applied decontamination treatment resulted in carcasses with the least visible

contamination. Trimmed (T), hot water (HW), ozone (OZ) and peroxide (PER) treatments resulted in visual scores which were higher than those for the TW treatment, but were lower ($P < 0.05$) than that achieved with washing (W) only. Conventional washing without trimming was the least effective treatment in removing visible contamination from the carcasses.

Table 2.2.10. Visual scores for cleanliness of intentionally contaminated carcasses before and after application of decontamination treatments (Reagan et al., 1996)

Treatment [a]	Mean visual score [b]	No. carcasses treated	SD of mean
Before treatment			
Control (CNT)	3.51A	144	0.65
Trimmed (T)	3.47A	144	0.50
Washed (W)	3.39A	144	0.50
Trimmed and washed (TW)	3.42A	144	0.49
Hot-water washed (HW)	3.33A	48	0.52
Hydrogen peroxide (PER)	3.47A	47	0.50
Ozone (OZ)	3.52A	48	0.55
After treatment			
Control (CNT)	3.44A	144	0.70
Trimmed (T)	0.47D	144	0.88
Washed (W)	1.14B	144	0.78
Trimmed and washed (TW)	0.16E	144	0.35
Hot-water washed (HW)	0.54D	48	0.46
Hydrogen peroxide (PER)	0.85C	47	0.68
Ozone (OZ)	0.66CD	48	0.55

[a] See text and Table 2.2.09 for a description of treatments.

[b] Means within a scoring time followed by different letters are different ($P < 0.05$).

Washing only, or application of ozone (OZ) and hydrogen peroxide (PER) rinses, resulted in average reductions in bacterial populations of approximately 1 log unit (Table 2.2.11). Although the application of the two chemical interventions, OZ and PER, resulted in lower ($P < 0.05$) average microbiological populations than the control samples, the means were not significantly ($P > 0.05$) different from those achieved with conventional washing or trimming. It was interesting to note the relatively large populations of bacteria remaining on the surface after the treatments, even though the scores for visible contamination on carcasses treated by several of these interventions were quite low.

Table 2.2.11. Populations of aerobic bacteria and *Escherichia coli* Biotype I on beef carcasses which were intentionally contaminated and then decontaminated with specific intervention treatments (Reagan et al., 1996)

Bacteria counted (treatment [a])	Mean counts [b]	No. carcasses treated	SD of mean
Aerobic plate count			
Control (CNT)	4.20A	142	1.32
Trimmed (T)	2.88C	142	1.10
Washed (W)	3.24B	144	1.15
Trimmed and washed (TW)	2.35D	144	0.99
Hot-water washed (HW)	2.20D	46	0.69
Hydrogen peroxide (PER)	3.06BC	48	1.09
Ozone (OZ)	2.90BC	48	1.04
***E. coli* Biotype I**			
Control (CNT)	2.23A	142	1.22
Trimmed (T)	0.62C	142	0.69
Washed (W)	1.19B	144	0.99
Trimmed and washed (TW)	0.56C	144	0.59
Hot-water washed (HW)	0.41C	48	0.28
Hydrogen peroxide (PER)	1.25B	47	0.80
Ozone (OZ)	1.09B	48	0.90

[a] See text and Table 2.2.09 for a description of treatments.

[b] Aerobic plate counts, CFU/cm^2; *E. coli,* MPN/cm^2. Means followed by different letters are different ($P < 0.05$).

The mean populations of *E. coli* Biotype I followed the same general trend as did the populations of aerobic bacteria (Table 2.2.11), with the lowest populations obtained with the TW, HW, and T treatments. As with the aerobic plate counts, the HW treatment resulted in the least carcass-to-carcass variation, as was indicated by the lower standard deviation. Although the trimming-only (T) treatment resulted in statistically ($P < 0.05$) lower populations of aerobic bacteria than the W treatment and higher than the TW treatment, T was not significantly ($P > 0.05$) different from TW for *E. coli* counts. Use of these treatments (T, TW, HW) resulted in an average reduction in populations of approximately 1.7 log units when compared to the control. As was the case with the aerobic bacteria, the W, OZ, and PER treatments resulted in significant ($P < 0.05$) reductions in *E. coli* counts compared to the control.

All of the processing treatments -- trimming, washing, and trimming and washing -- significantly ($P < 0.05$) reduced the incidence of *Listeria spp.* and *Salmonella spp*. on the carcasses (Table 2.2.12). Trimming and washing resulted in the lowest incidence of these two bacteria of potential public health significance, although the individual treatments could not be statistically differentiated.

Table 2.2.12. Incidence of bacteria of public health significance on samples from beef carcasses which had been intentionally contaminated and then decontaminated with specific intervention treatments (Reagan et al., 1996)

Treatment [b]	Number of samples (positive/total) [a]		
	Listeria spp.	***Salmonella spp.***	***E. coli* O157:H7 [c]**
Control (CNT)	61/142	43/142	1/142
Trimmed (T)	35/140*	11/142*	3/142
Washed (W)	39/143*	13/144*	1/144
Trimmed and Washed (TW)	18/143*	2/144*	2/144
Hot-water washed (HW)	15/45	1/46*	0/46
Hydrogen peroxide (PER)	16/47	15/47	0/47
Ozone (OZ)	11/48*	19/48	0/48

[a] Treatment values within a genus marked with asterisks (*) are significantly ($P < 0.05$) different from the control treatment value.

[b] See text and Table 2.2.09 for a description of treatments.

[c] *E. coli* O157:H7: insufficient number of positive samples to determine treatment differences.

The current industry practice of trimming and washing reduced the incidence of *Listeria spp*. from 43.7% to 12.6%, and reduced the incidence of *Salmonella spp.* from 30.3% to 1.4%. Although the initial incidence levels of these two bacteria seem high, these carcasses were deliberately contaminated to obtain sufficiently high counts for statistical analysis and are not typical of the average cattle being processed. Hot water washing also significantly ($P < 0.05$) reduced the incidence of *Salmonella spp*. when compared to that of the control, but the reduction of *Listeria spp.* incidence was not significant ($P > 0.05$). PER and OZ treatments also reduced the incidence of pathogens and their effect should have included any residual activity, since sample analysis was conducted on the day after treatment. The total number of samples that were positive for *E. coli* O157:H7 was insufficient to compare treatments and/or to differentiate for their ability to reduce the level of *E. coli* O157:H7 contamination. However, the data suggested that none of the treatments could be relied upon to completely eliminate that pathogen (*E. coli* O157:H7) from the carcasses.

Conclusions: Ozone and hydrogen peroxide treatments, as applied in this study, had only minor effects and were approximately equivalent to conventional washing in reducing bacterial populations on beef.

2.2.6 Gorman et al. (1997) – Beef Washing and Storage

Abstract: Spray-washing reduced aerobic plate counts (APC) by 0. 88 to 2.83 log colony-forming units (CFU)/cm^2, with hot water (74EC) being the most effective treatment. Counts exceeded 6 log CFU/cm^2 in 1-3, 7-11, 11-16, 16-23 and 23-29 days of storage for unwashed, washed with hydrogen peroxide, washed with 35EC water or ozonated water or trimmed/washed with 35EC water, washed with commercial sanitizer, and washed with trisodium phosphate,

respectively. Samples washed with acetic acid or water of 74EC reached only 4.31 and 4.36 log CFU/cm^2, respectively, at 29 days of storage. Increases in the concentration of thiobarbituric acid reactive-substances (TBARS) were slowest in samples washed with trisodium phosphate. Spray washing with 2% acetic acid or 74EC water were the most effective treatments for reducing microbial growth, followed by trisodium phosphate which also reduced lipid oxidation during storage of beef.

Materials and Methods

Inoculum Preparation: A pure culture of streptomycin-resistant *Escherichia coli* (ATCC 11370) was prepared in nutrient broth (containing 625 mg of dihydrostreptomycin/mL at 35EC for 24 h. A dilution of the Culture (10^8 CFU/g) then was mixed with fresh bovine feces (randomly collected each day of experimentation from the holding pens of a large commercial beef slaughter facility) in a sterile Whirl-Pak bag by hand massaging for 2 min. The objective of this procedure was to obtain a high and consistent inoculum, and a desired fecal paste consistency, which was achieved by addition of sterile water to the inoculated fecal material to reach a ratio of three parts of total added liquid, to one part of fecal material. The inoculum then was transported from the laboratory, in coolers with ice packs, to a commercial slaughtering/dressing facility for inoculation of samples and subsequent spray-washing within the same day.

Sanitizing Agents: Sanitizing agents tested by spraying them on beef samples included 2% acetic acid, 12% trisodiurn phosphate, 5% hydrogen peroxide (30% stock solution), 0.5% ozonated water, and 0.3 % of a commercial sanitizer (RPM Acid Sanitizer) consisting of decanoic acid 3%, nonanoic, acid 3%, phosphoric acid 8.5%, sulfuric acid 9.5%, propionic acid 10% and inert ingredients 66%.

The sanitizing agent solutions were prepared by mixing tap water with the specified chemical within 24 h of use and holding them in closed containers in a cold room (4EC) until used. The solutions of water plus chemical sanitizers were sprayed on the samples at 16EC, 1.38 bar and at exposure times of 12 s (equivalent to 300 carcasses/h), after the samples were spray-washed with water (35EC) at 20.68 bar for 12s.

Sample Handling and Decontaminating Treatments: A sample of adipose tissue (approximately 1.25 cm thick) from the outside surface of the chuck of beef carcasses was removed using a sterile knife-blade, prior to any routine carcass washing or trimming (< 15 min postmortem), and was transported to a room adjacent to the slaughtering/dressing area of the packing plant using plastic trays cleaned with an alcohol spray. Each piece of adipose tissue was cut aseptically with a knife blade to form a 10 cm x 10 cm portion and inoculated by use of sterile plastic inoculating loops. Inoculating loops were immersed into the fecal paste and the inoculum was transferred to the center of each square of adipose tissue to achieve a 2.5 cm^2 area with contamination on the side with intact fascia. The 2.5 cm^2 area was obtained using a 0.625 cm^2 inoculating loop and transferring inoculum to the adipose tissue for the appropriate number of times (n = 4).

After inoculation, each piece of adipose tissue was held for 15 min at room temperature (20EC) to allow for attachment of fecal material and bacteria. The pieces of adipose tissue then were

treated by hand trimming or spray washing at the specified conditions. Spray washing was done in a specially designed, test-size, conveyorized, model spray washing cabinet with one 0.3125 cm (MEG 2150) diameter oscillating nozzle; the nozzle oscillation was set at 80 rpm and the oscillation pattern covered the entire length of the piece of adipose tissue being washed at a distance of 12 cm. The cabinet was custom-made for these studies and designed to simulate slaughter production-speed as well as the wash-action of a final carcass wash, spray-washing cabinet. In addition to rinsing, with the different sanitizing agents, samples that had been spray washed with water, other samples were spray-washed with water at 35EC or 74EC for 12 s (300 carcasses/h) at 20.68 bar. Additional samples were trimmed with a sterile knife and forceps, cutting vertically to remove all visible fecal contamination, and the samples subsequently were spray washed (35EC, 20.68 bar, 12 s). The knife and forceps were decontaminated between samples using alcohol and a flame.

Packaging and Visual Evaluation: Following the spray-washing treatment, each piece of adipose tissue was placed onto a 10 cm x 10 cm Styrofoam tray and overwrapped with polyvinyl chloride film. The samples then were placed in coolers containing ice packs; and, after all treatments were completed, the samples were stored in a cold room (4EC) under 350 lux of continuous, cool white fluorescent illumination. Duplicate samples were randomly obtained for each of the treatments on days 1, 3, 7, 11, 16, 23 and 29, and evaluated for total populations of aerobic microorganisms and for content of thiobarbituric acid-reactive substances (TBARS).

Analyses: Total aerobic plate counts (APC) were determined by aseptically cutting a 3 cm by 3 cm square from the center of each piece of adipose tissue, approximately 1.25 cm thick, and transferring the tissue into a sterile stomacher bag containing sterile 0.1% peptone water (Difco). The samples then were macerated for 2 min using a model 400 stomacher. Duplicate samples were serially diluted with sterile 0.1% peptone water and spread-plated on tryptic soy agar (Difco). The inoculated plates were incubated for 24 h at 35EC. The colonies then were counted, recorded and results were expressed as log CFU/cm^2.

Statistical Analysis: A completely randomized design was used in this study with dependent variables of total aerobic counts, thiobarbituric acid-reactive substances, and visual evaluation scores for the center area and the surrounding areas. Independent variables consisted of treatment, replication, day, and all possible interactions of the above. The study was replicated twice and duplicate samples were analyzed each time. The data were analyzed by analysis of variance using the General Linear Models procedure of SAS. Least squares means (LSM) were calculated with n=4 (except ozone, n=2). The LSM were separated using the Least Significant Difference procedure. The alpha level was set at 0.05 throughout the study.

Results And Discussion: The treatment-by-day interaction was not significant ($P > 0.05$) for APC; however, differences ($P<0.05$) were noted for the individual effects of treatments and days of storage. Inoculation increased contamination by 1.82 log CFU/cm^2, while treatments (during their application) reduced microbiological population levels compared to the inoculated/unwashed control, by 0.88 to 2.83 log CFU/cm^2, with the most effective treatment being hot (74EC) water (Table 2.2.13).

The 74EC plain water spray-washing treatment and the acetic acid treatment were the most effective ($P > 0. 05$) in reducing microbial growth during sample storage. The maximum APC

reached by these treatments during the 29 days were 4.36 and 4.31 CFU/cm^2, respectively (Table 2.2.13). Times at which samples exceeded 6 log CFU/cm^2 were 7-11 days for hydrogen peroxide; 11-16 days for samples treated with 35EC water, ozonated water, or trimmed and washed with 35EC water; 16-23 days for samples treated with commercial sanitizer; and, 23-29 days for samples treated with trisodiurn phosphate. In general, the most effective decontamination treatments in terms of inhibiting microbial growth during product storage were hot (74EC) water and acetic acid (2%), followed by trisodium phosphate (12%). The other treatments had a lesser effect on rate of microbial growth, which was similar to that on uninoculated/unwashed samples.

The TBARS values for samples from all treatments gradually increased during the 29-day storage period with the F-test being significant ($P < 0.05$) for the treatment-by-day interaction (Table 2.2.14). There was no difference ($P > 0.05$) among samples from different treatments, in the TBARS values on sampling days 1, 3 and 7. Samples treated with trisodium phosphate (12%) reached a maximum value of 3.94 mg malonaldehyde/kg of wet tissue on day-29 of storage, whereas the uninoculated/-unwashed samples reached a level of 4.27 mg/kg of wet tissue on day-23. Samples of spray-washed adipose tissue from the other treatments sustained more rapid increases in TBARS during storage than did samples treated with trisodium phosphate.

Table 2.2.13. Effects of spray-washing treatments on microbial growth (log CFU/cm^2) on samples of beef adipose tissue during 29 days of aerobic storage (4EC) (Gorman et al., 1997)

Day	Uninoculated/ Unwashed	Inoculated/ Unwashed	Inoculated/ Trimmed/ Washed (35C)	Water Wash (35C)	Water Wash (74C)	Acetic Acid (2%)	Ozonated Water (0.5%)	Hydrogen Peroxide (5%)	Trisodium Phosphate (12%)	Commercial Sanitizer (0.3%)[y]
1	2.97(0.80)^{abD}	4.79(2.02)^{aB}	3.91(0.80)^{abC}	3.70(0.74)^{abB}	1.96(0.25)^{bB}	3.50(0.72)^{abAB}	3.30(0.04)^{abB}	2.93(0.51)^{abB}	3.10(0.51)^{abC}	2.92(0.32)^{abD}
3	4.02(1.72)^{bCD}	6.86(0.34)^{aA}	2.52(1.14)^{bC}	3.95(0.40)^{bB}	2.47(0.81)^{bB}	2.38(0.84)^{bAB}	4.52(0.22)^{abB}	3.54(0.65)^{bB}	2.51(1.31)^{bC}	3.57(0.72)^{bD}
7	4.80(2.62)^{abCD}	7.16(0.97)^{aA}	3.91(1.60)^{bcC}	3.70(0.53)^{bcB}	2.26(0.93)^{cB}	2.31(0.86)^{cB}	5.00(0.64)^{abAB}	3.80(1.49)^{bcB}	4.13(1.68)^{bcBC}	4.45(1.58)^{bCD}
11	4.40(2.11)^{abCD}	7.67(0.90)^{aA}	4.28(1.23)^{abBC}	4.49(1.66)^{bcAB}	4.35(2.08)^{cAB}	3.53(0.75)^{cAB}	5.43(0.43)^{abcAB}	6.40(1.06)^{abA}	4.44(1.76)^{bcBC}	4.72(2.00)^{bcBCD}
16	5.90(2.29)^{abABC}	6.98(1.36)^{aA}	6.11(2.68)^{abAB}	6.07(0.55)^{abA}	3.14(1.86)^{cAB}	4.24(2.32)^{bcAB}	7.59(1.20)^{aA}	6.07(0.54)^{abA}	3.80(1.52)^{bcBC}	5.90(1.73)^{abABC}
23	6.85(2.35)^{abA}	8.74(0.46)^{aA}	7.66(2.31)^{abA}	6.23(1.60)^{bcdA}	4.61(2.94)^{cbA}	3.48(1.36)^{cAB}	7.68(1.14)^{abA}	6.67(0.93)^{bcA}	5.24(1.20)^{abcAB}	6.82(0.63)^{abcA}
29	6.31(1.45)^{bcdAB}	9.11(0.34)^{aA}	8.02(0.45)^{abA}	5.87(1.26)^{bcdAB}	4.36(1.80)^{cdAB}	4.31(1.43)^{dA}	---[z]	6.83(1.54)^{abA}	7.04(1.10)^{abA}	6.59(1.49)^{bcAB}

[a-c] Means (standard deviations) in the same row with unlike superscripts are different at $P<0.05$

[A-D] Means (standard deviations) in the same column with unlike superscripts are different at $P<0.05$

[y] Commercial sanitizer consists of 3% decanoic acid, 3% nonanoic acid, 8.5% phosphoric acid, 9.5% sulfuric acid, 10% propionic acid, and 66% inert ingredients

[z] Data were not collected

Table 2.2.14. Effect of spray-washing treatments on thiobarbituric acid reactive substances (mg of malonaldehyde/kg of wet tissue) in samples of beef adipose tissue during 29 days of aerobic storage (4EC) (Gorman et al., 1997)

Day	Uninoculated/ Unwashed	Inoculated/ Unwashed	Inoculated/ Trimmed/ Washed (35C)	Water Wash (35C)	Water Wash (74C)	Acetic Acid (2%)	Ozonated Water (0.5%)	Hydrogen Peroxide (5%)	Trisodium Phosphate (12%)	Commercial Sanitizer (0.3%)[y]
1	0.33(0.03)^{D}	0.36(0.10)^{C}	0.36(0.04)^{D}	0.41(0.11)^{C}	0.47(0.03)D	0.59(0.15)^{D}	0.42(0.15)^{B}	0.64(0.31)^{D}	0.39(0.12)^{C}	0.39(0.13)^{C}
3	0.53(0.04)^{D}	0.52(0.09)^{C}	0.72(0.08)^{CD}	0.59(0.08)^{C}	0.74(0.32)^{D}	0.82(0.13)^{D}	0.82(0.16)^{B}	0.75(0.04)^{D}	0.51(0.09)^{C}	0.58(0.10)^{C}
7	0.88(0.11)^{CD}	1.05(0.23)^{C}	1.53(0.72)^{CD}	0.94(0.16)^{C}	1.23(0.43)^{CD}	1.50(0.19)^{CD}	1.33(0.30)^{B}	1.33(0.15)^{D}	1.03(0.45)^{BC}	1.30(0.23)^{C}
11	1.95(0.67)^{abC}	1.28(0.38)^{bC}	1.69(0.48)^{abC}	1.63(0.42)^{abBC}	2.47(0.79)^{abC}	2.69(0.49)^{aC}	2.21(0.53)^{abB}	2.64(0.58)^{aC}	1.84(0.75)^{abB}	2.74(0.69)^{aB}
16	3.33(1.79)^{bcB}	1.46(0.23)^{dC}	3.35(1.18)^{bcB}	2.62(1.14)^{cdB}	4.26(0.19)^{abB}	4.13(0.77)^{abB}	2.02(0.17)^{cdB}	5.31(0.80)^{aB}	2.09(0.33)^{cdB}	2.78(1.22)^{aB}
23	4.27(3.17)^{abcB}	3.01(1.50)^{cB}	4.69(1.90)^{abA}	5.76(1.33)^{aA}	4.91(1.05)^{abB}	5.68(1.00)^{aA}	5.77(0.11)^{aA}	4.91(0.72)^{abB}	3.79(0.74)^{bcA}	5.36(2.18)^{aA}
29	6.82(3.03)^{abcA}	5.28(0.18)^{cdA}	6.12(0.92)^{bcA}	1.96(0.12)^{eBC}	7.80(0.47)^{aA}	6.51(0.55)^{abcA}	—[z]	7.11(1.17)^{abA}	3.94(0.35)^{dA}	6.14(1.51)^{bcA}

$^{a-e}$ Means (standard deviations) in the same row with unlike superscripts are different at P<0.05

$^{A-D}$ Means (standard deviations) in the same column with unlike superscripts are different at P<0.05

[w] Results on days 1, 3, and 7 are not significantly different among samples in any treatment; therefore, they do not have superscripts

[y] Commercial sanitizer consists of 3% decanoic acid, 3% nonanoic acid, 8.5% phosphoric acid, 9.5% sulfuric acid, 10% propionic acid, and 66% inert ingredients

[z] Data were not collected

Based on these data, spray-washing with hot water or acetic acid not only reduced initial contamination, but also increased the microbial shelf life of beef adipose tissue stored in an aerobic environment. Other spray washing treatments reduced the rate of microbial growth only to those levels inherent to uninoculated/unwashed samples. Among control and spray washed samples, those treated with trisodium phosphate had the lowest level of malonaldehyde per kg of wet tissue.

SUMMARY OF RED MEAT PROCESSING SECTION

Antimicrobial effects of ozone on beef carcasses were reported in six major investigations in 1968,1989, 1990, 1995, 1996, and 1997.

Gaseous ozone (0.6 mg/m^3) in air and ozone in 11% CO_2 at 98% Equilibrium Relative Humidity slightly delayed surface growth of *Pseudomonas, Candida, Penicillium*, and *Thamnidium* on stored carcasses, but sub-surface growth in crevices was not inhibited. Optimal conditions for muscle stored at 0.3EC were obtained by applying a continuous ozone concentration of 0.6 mg/m^3.

Ozonated water (0.3 to 2.3 mg/L ozone at 35EC) rinse and hydrogen peroxide (5%, 35EC) rinse reduced surface microbial counts (*Salmonella, Listeria, E. coli*, and APC) about the same as hot (74 to 87.8EC) water rinse, i.e., on the order of 3 log CFU/cm^2, when applied for 12 seconds. When the first spray washing treatments of 16 or 35EC plain water were followed chemical solution interventions, the most effective sanitizing agents were hydrogen peroxide (5%) and

ozonated water (0.5 mg/L), achieving reductions in total plate counts of 2.60 to 2.87, and 2.72 to 2.86 CFU/cm^2, respectively.

TBA values were similar after storage for 28 days at 4EC for carcasses washed with water, acetic acids, ozone water, and peroxide water. The data indicated little or no consistent effect of the chemical interventions on development of oxidative by-products.

Petitioners Comment

Based on these six investigations, treatment of beef carcasses using high pressure (68 bar) spraying with hot (74EC) 5% hydrogen peroxide for 12 seconds followed by low pressure (20.68 bar) cool ozone water (0.5 mg/L at 16EC) for 12 seconds is likely to produce best surface count reduction and modest shelf life extension. The impact of microbial population selection needs further evaluation, particularly hot water (87.6EC) that will select for heat resistant survivors, such as spore formers, e.g., *Clostridium botulinum* in subsurface crevices. Regrowth of such survivors could increase the risk of pathogenic microorganisms in the final product.

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.

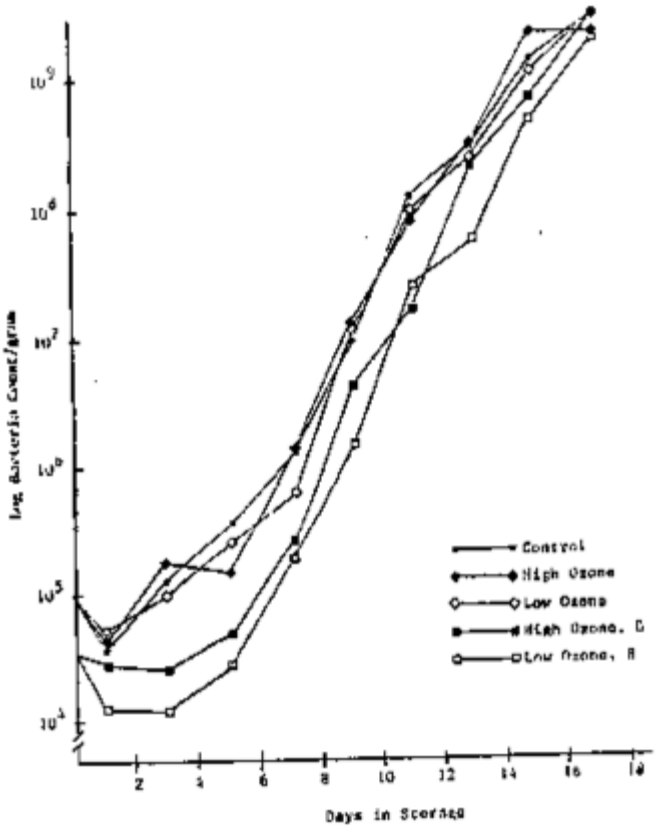


Figure 2.3.01. Log bacteria count vs days in storage for College Station phase of study (DeWitt et al., 1984)

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.

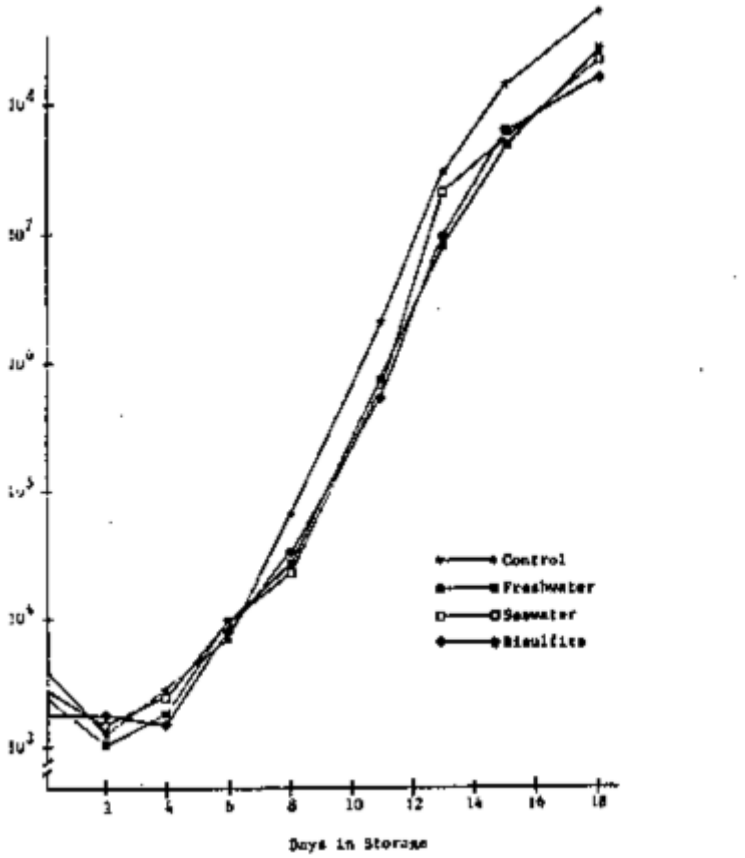


Figure 2.3.02. Log bacteria count vs days in storage for Corpus Christi phase of study (DeWitt et al., 1984)

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.

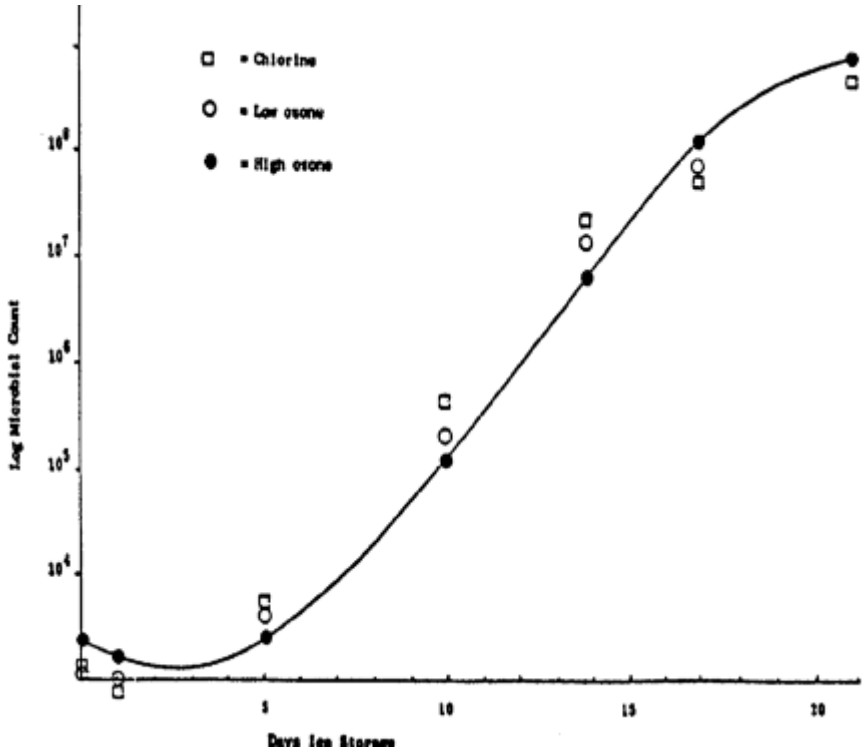


Figure 2.3.03. Microbial counts of sockeye salmon stored in treated ice (data in Table 2.3.09) (Lee and Kramer, 1984).

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.

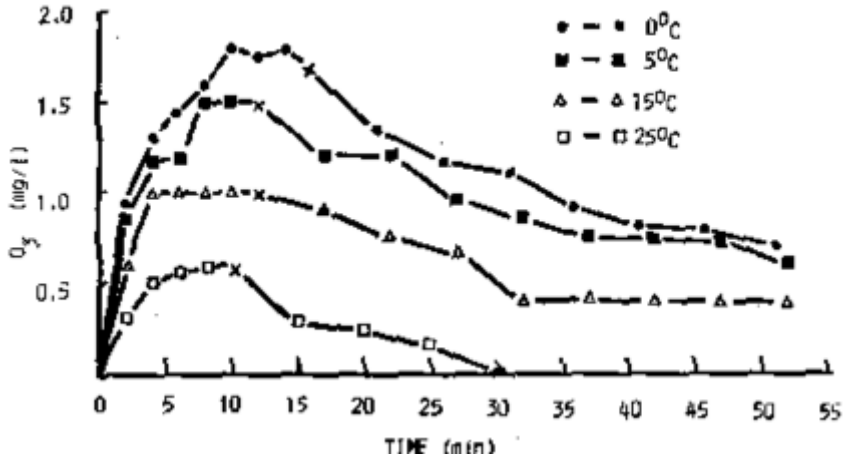


Figure 2.3.04. Ozone concentrations in distilled water at different temperatures during and after flushing with ozone. "X" indicates the time when ozone flushing was ceased (Chen et al., 1987).

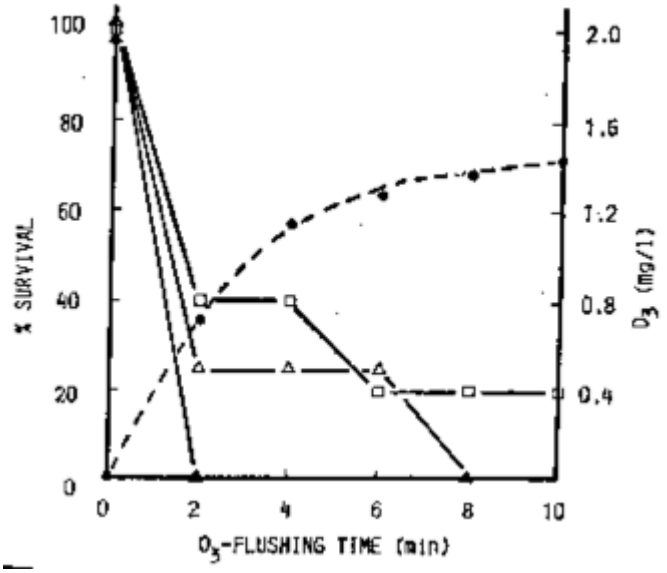


Figure 2.3.05. Sterilization effect of ozone-treated distilled water at 5EC on *Vibrio cholerae, Escherichia coli, Salmonella typhimurium* (>), *Vibrio parahaemolyticus* (♠) and *Staphylococcus aureus* (9). Ozone concentration indicated as (X) (Chen et al., 1987).

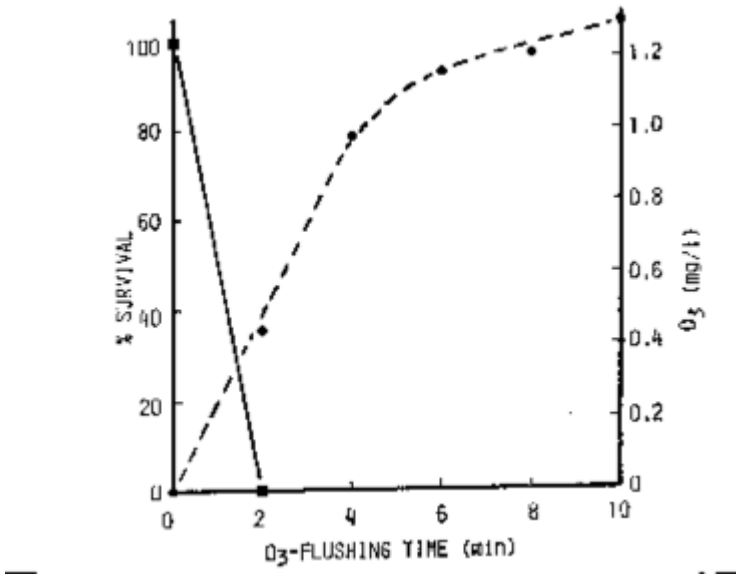


Figure 2.3.06. Sterilization effect of ozone-treated distilled water at 25EC on *Vibrio cholerae, Escherichia coli, Salmonella typhimurium, S. aureus* ( ). Ozone concentration indicated as (X) (Chen et al., 1987).

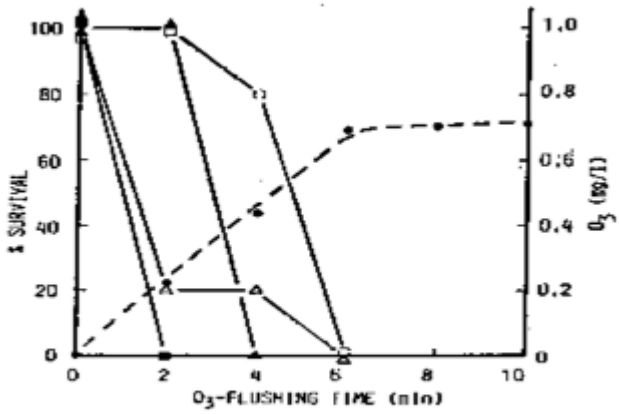


Figure 2.3.07. Sterilization effect of ozone-treated water mixed with 300 ppm milk powder at 5EC on *Vibrio cholerae* (♠), *Vibrio parahaemolyticus*, *Staphylococcus aureus* ( ), *Escherichia coli* (~), and *Salmonella typhimurium* (>). Ozone concentration indicated as (X) (Chen et al., 1987).

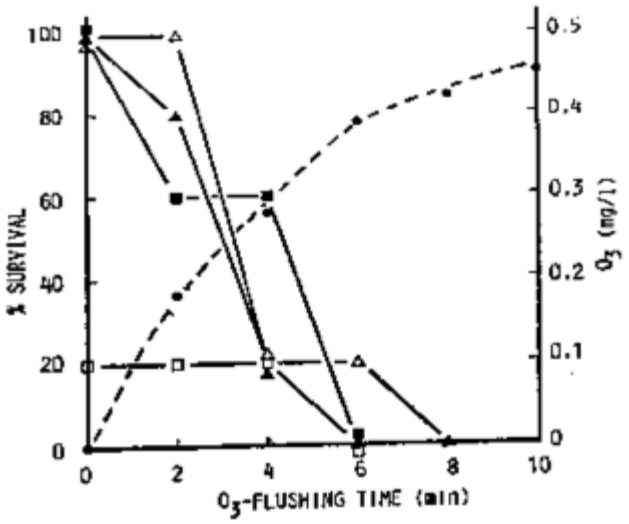


Figure 2.3.08. Sterilization effect of ozone-treated water mixed with 300 ppm milk powder at 25EC on *Vibrio cholera*, *Salmonella typhimurium* (>), *Escherichia coli* (♠), *Vibrio parahaemolyticus* (9),and *Staphylococcus aureus* ( ). Ozone concentration indicated as (X) (Chen et al., 1987).

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.

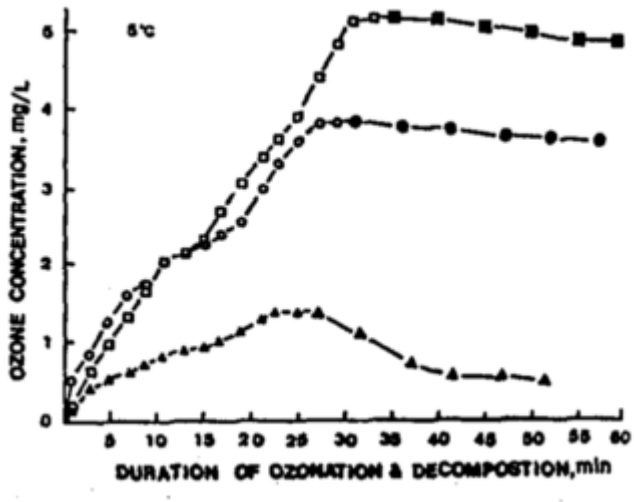


Figure 2.3.09. Solubility and decomposition of ozone in distilled water (", !), 2% NaCl solution (G, #) and shrimp meat extract (♠, >), when the 5EC liquids were ozonated at a flow rate 100 mL/min. Solid marks indicated ozone flushing was stopped and ozone was decomposed.

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).

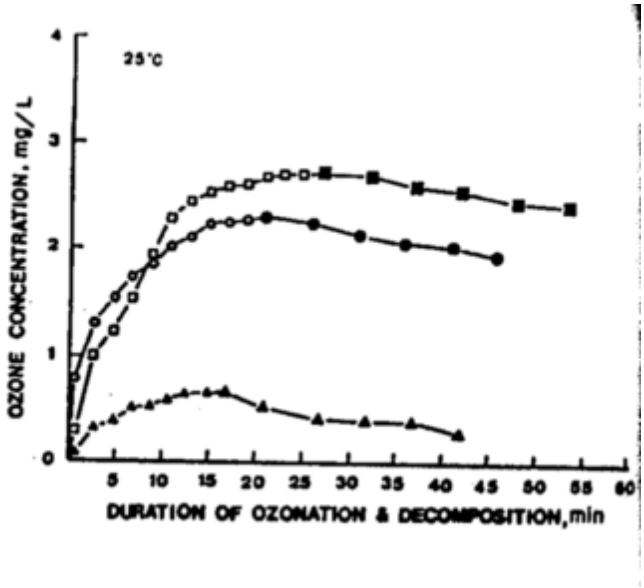


Figure 2.3.10. Solubility and decomposition of ozone in distilled water (", !), 2% NaCl solution (G, #) and shrimp meat extract (♠, >), when the 25EC liquids were ozonated at a flow rate 100 mL/min. Solid marks indicated ozone flushing was stopped and ozone was decomposed.

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.

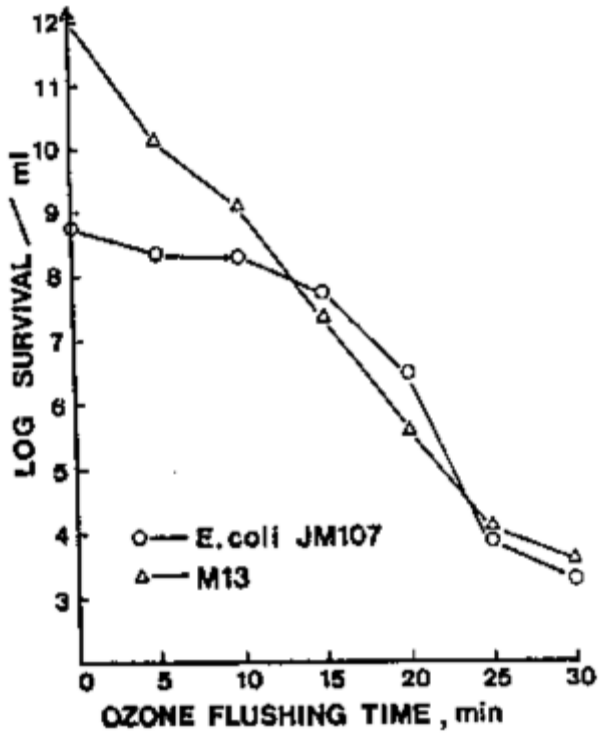


Figure 2.3.11. Effect of ozonation on the survival of E. coli JM107 and phage M13 in M9 medium which was ozonated with a flow rate of 100 mL/min, to obtain a final concentration of 5 mg-ozone/mL within 30 min. (Chen et al., 1992).

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.

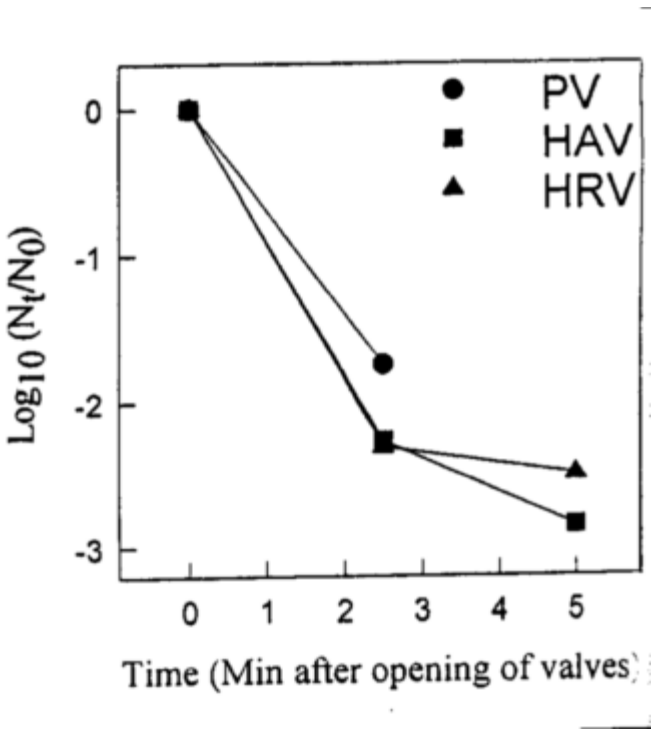


Figure 2.3.12. Inactivation of viruses in shellfish by cooking. PV poliovirus; HRV human rotavirus; HAV hepatitis A virus (Abad et al., 1997.

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.

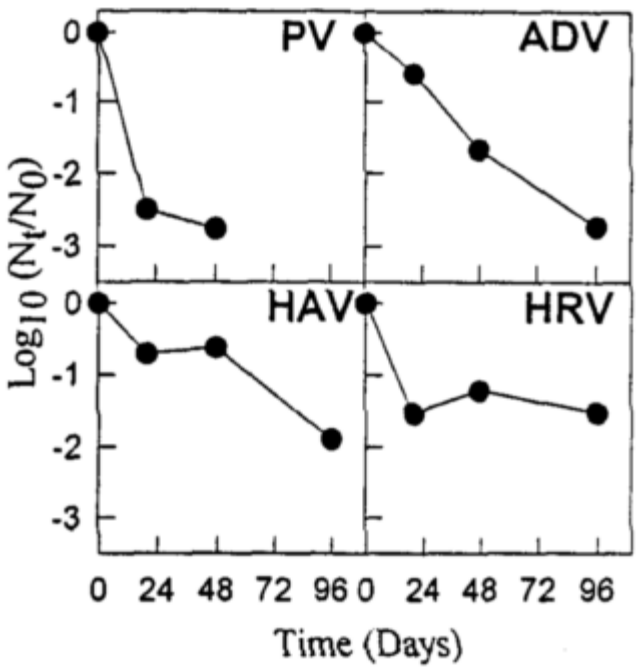


Figure 2.3.13. Removal of viruses in shellfish by depuration. PV poliovirus; ADV enteric adenovirus; HRV human rotavirus; HAV hepatitis A virus (Abad et al., 1997).

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.

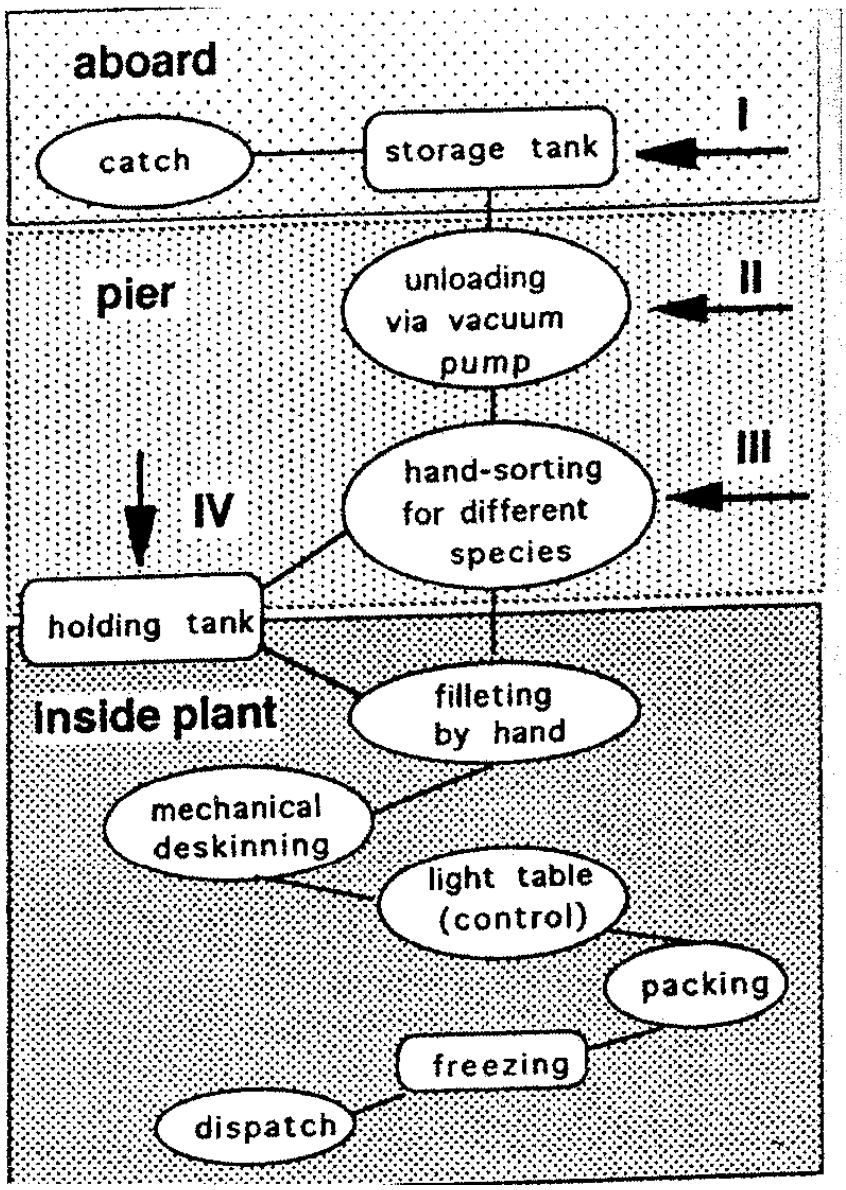


Figure 2.3.14. Flow chart of fish processing in a typical plant. Major steps of processing for fresh fish from catch to dispatch are indicated. Each step follows its own time course. Fish may be stored for various time periods after being pumped ashore. Catch usually is cooled in ice-water near 0EC. 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.

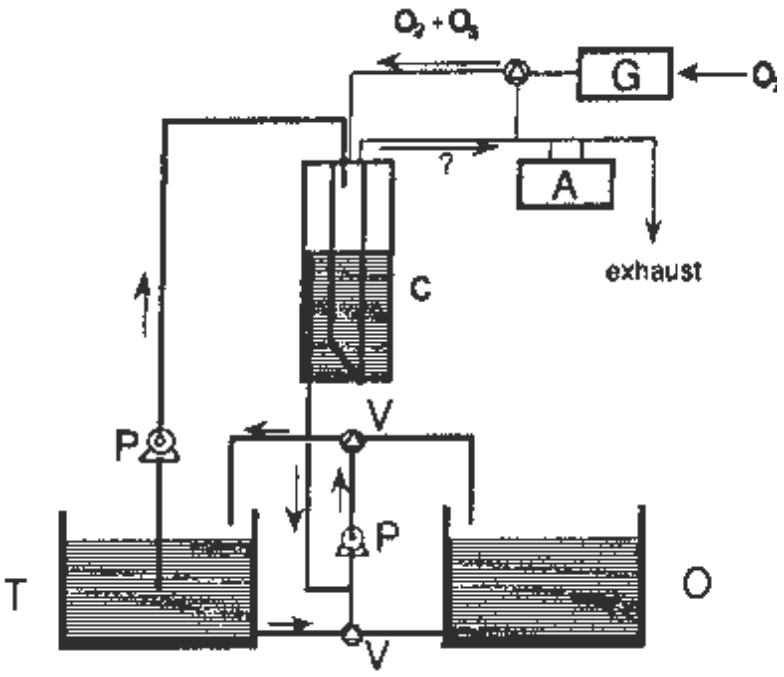


Figure 2.3.15. Schematic diagram of the experimental unit for simulating storage of fish on board a fishing vessel; G: ozone generator; A: ozone analyzer; C: Contacting column; P: Pump; V: 3-way valve: T: ozonated tank; O: oxygenated control; arrows indicate direction of flow (Kötters et al., 1996)

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.

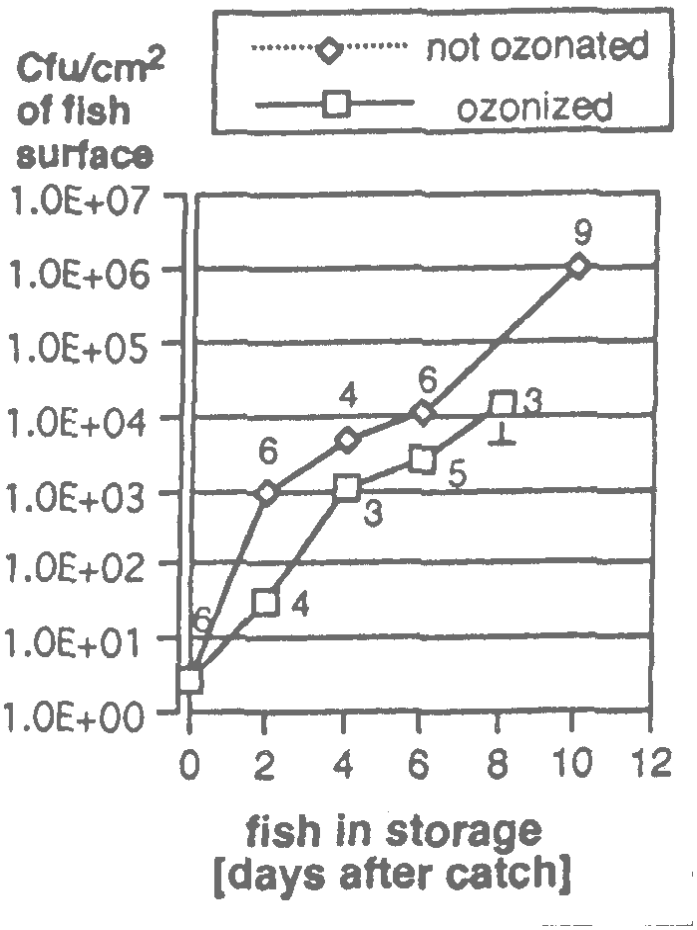


Figure 2.3.16. Comparison of psychrotrophic microbial growth on ozonized and control fish between capture and unloading at the processing plant wharf. Numbers = sample size, bars = standard deviation (Kötters et al., 1996).

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.

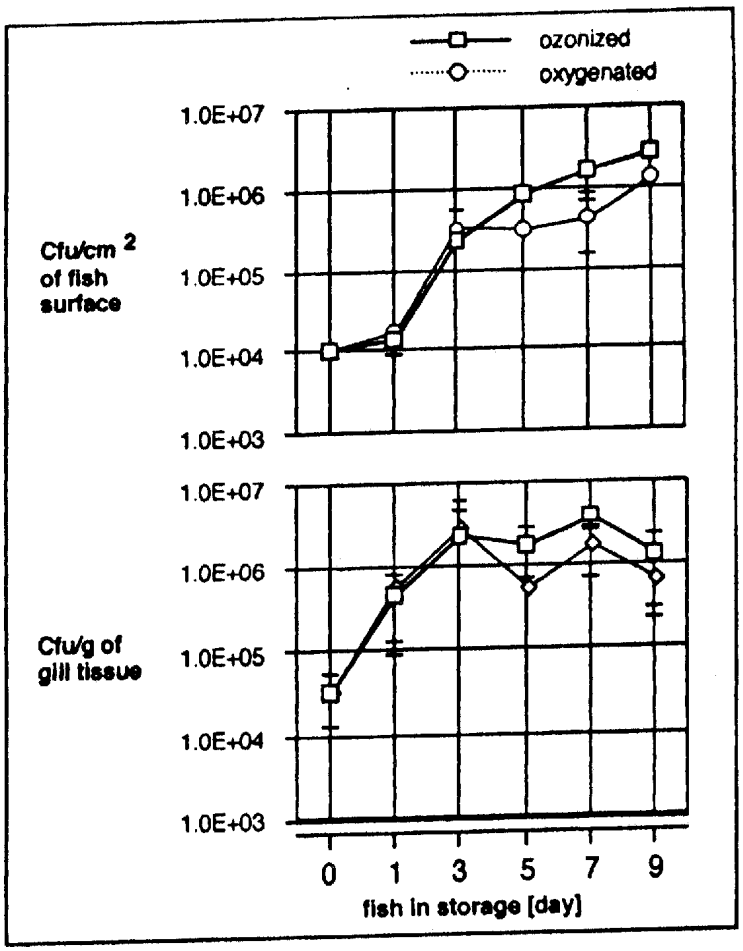


Figure 2.3.17. Microbiological changes of ozonized fish landed. Microbial counts from surface samples of fish indicated the number of microorganisms attached on the fish surface. Counts decreased during handling (pumping) of fish from the boat to the processing plant (Kötters et al., 1996).

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.

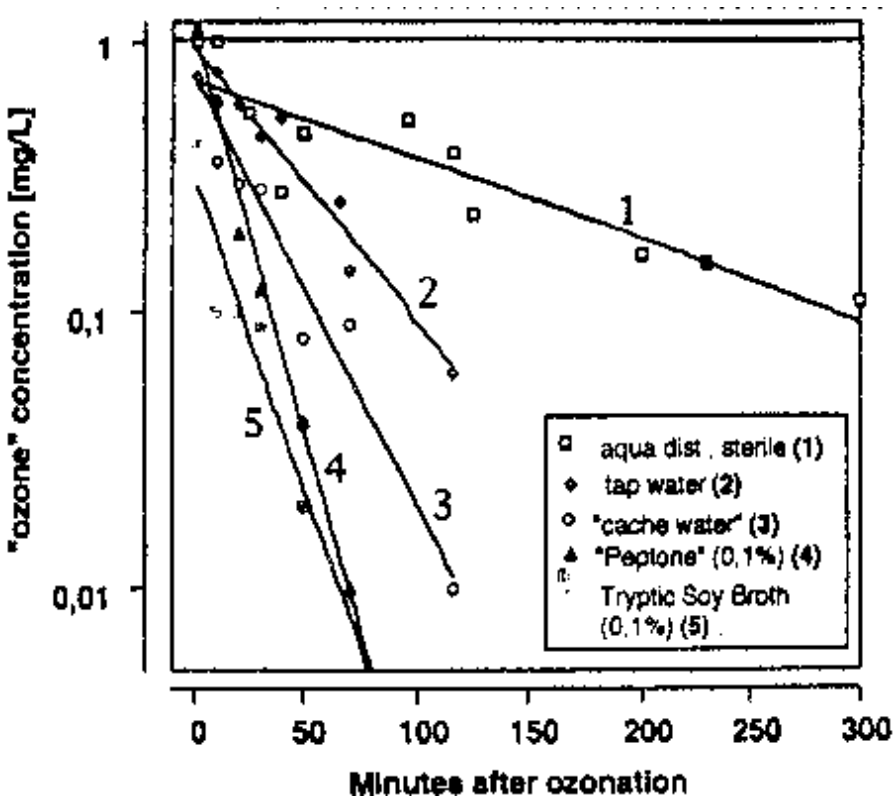


Figure 2.3.18. Simulated medium-scale storage offish in ozonized (30 min per day) and oxygenated (control) water. Effects on colony forming units (CFU) in gill tissue and skin surface samples. Number of determinations per sample point = 3; bars = standard deviation (Kötters et al., 1996).

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).

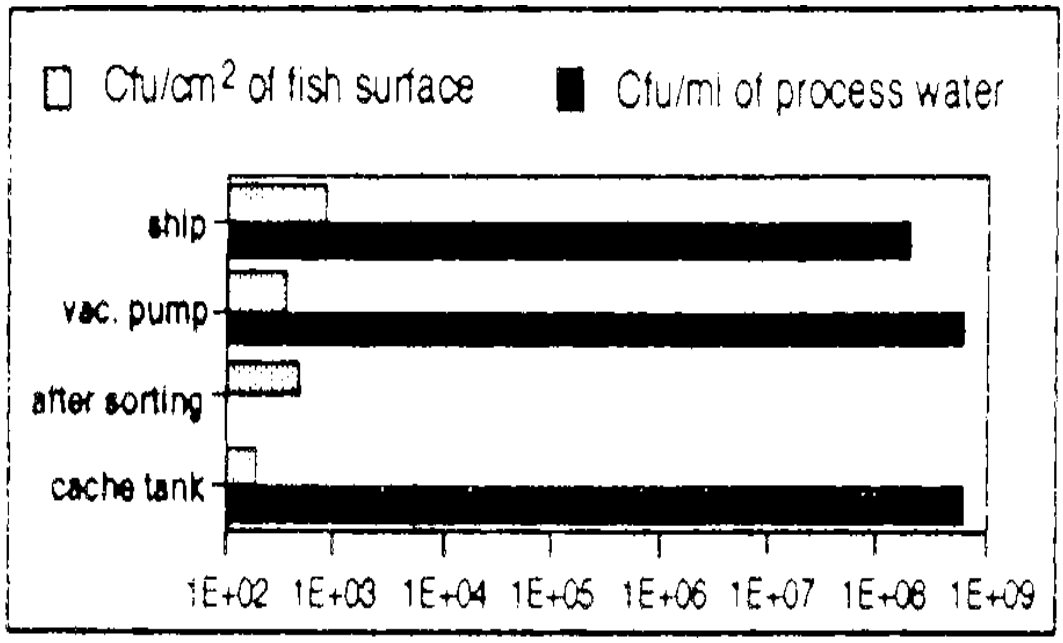


Figure 2.3.19. "'Ozone' (total radical oxidant) decomposition in different standard media (semi-logarithmic) at 20EC. 1=distilled water; 2=tap water (chlorinated). The data referring to media containing different organics are labeled "3-5". These are: 3=water from a fish processing plant holding tank; 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.

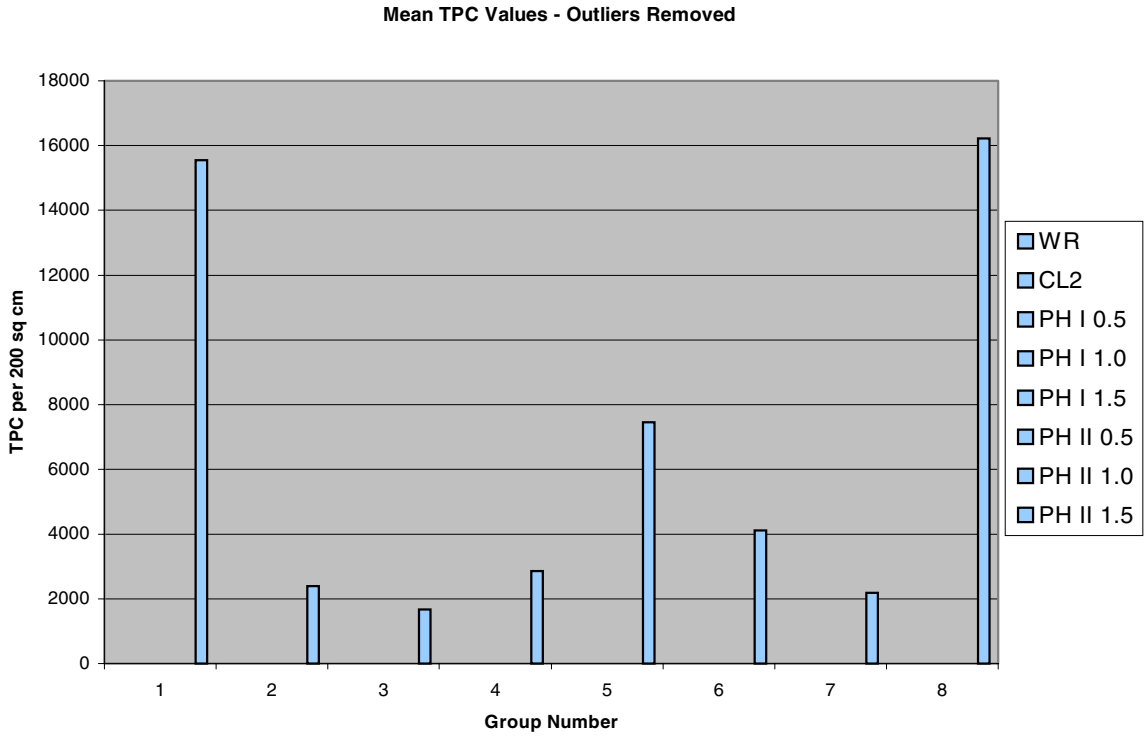


Figure 2.3.20. Mean TPC values – outliers removed (Goché and Cox (1999).

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.

2.4 FRUITS

2.4.1 Smock and Van Doren (1939) – Apples

Abstract: Some possibilities of the use of ozone in modified atmosphere chambers for the control of scald on McIntosh and Cortland apples is indicated by preliminary experiments. The storage atmosphere is first treated with a relatively high concentration of ozone and then this concentration is reduced to non-toxic amounts before it is returned to the chamber proper.

Since ozone has such a powerful oxidizing action, it seemed possible it might have some effect on the volatile esters which cause storage scald. In these preliminary experiments the amount of ozone was determined by taking a measured volume of air-ozone mixture and using the usual iodine-sodium thiosulfate titration method. Deozonizing was accomplished by passing the atmosphere through a long glass tube filled with small pieces of scrap rubber. The rate of air flow through all vessels in series was at the rate of 26 liters per hour.

Experiment A. Rhode Island Greening apples were held continuously at room temperature (about 70EF) in gas-tight containers in this experiment. One set of vessels was in the following order: concentrated ethyl acetate ≡ Rhode Island Greening apples ≡ suction. Artificially induced scald appeared on these fruits within 24 hr and became progressively worse. A second set of vessels was in the following series: concentrated ethyl acetate ≡ a mixing chamber in which there was a sealed-in ozone generator ≡ a deozonizer ≡ Rhode Island Greening apples ≡ suction. The amount of ozone in the air stream coming from the mixing chamber was 5 parts per million, but after passing through the deozonizer it was reduced to 1-2 parts per million. **At the end of 72 hr, no scald was apparent on these fruits.** The ozone supply then was shut off and scald appeared within 6 hr.

Experiment B. Experiment A was repeated with Yellow Newtown apples at 36EF. Within 3 days artificially induced scald appeared on the non-ozone lot. After the fourth day both lots were removed to room temperature. Within 12 hr, 100 per cent scald appeared on the non-ozone lot whereas none appeared on the ozone lot even after several days at room temperature.

Experiment C. Yellow Newtowns with a very green ground color were held for three months at 36EF. in two different series. In the first series the order of vessels was as follows: water 6 full ripe Duchess apples 6 Yellow Newtown apples6 suction. The second series was as follows: water 6 full ripe Duchess apples; 6 mixing chamber containing a sealed in ozone generator 6 deozonizer 6 Yellow Newtown apples 6 suction. The amount of ozone used was similar to that in Experiments A and B. **At the end of three months, the non-ozone Newtowns were covered with mold but had no apparent scald.** The ozone-treated Newtowns had no mold and were also free of scald. Both lots then were removed to room temperature. After three days the non-ozone Newtowns had 70 per cent scald and after 10 days had 80 per cent scald with 60 per cent of the total amount of scald of a very serious type. The ozone-treated Newtowns developed no scald during 13 days at room temperature when the experiment was discontinued. At this time the ground color on the non-ozone lot averaged 3.1, whereas it was only 2.5 on the ozone-treated Newtowns.

It is not known whether scald control was had in this case as a result of the effect of the ozone on vapors coming from the Duchess or from the effect of the ozone passing through the deozonizer and oxidizing esters arising from the Newtowns themselves. The deozonizer did not completely remove the ozone as has been indicated but reduced it to non-toxic concentrations. Previous trials had indicated that without the deozonizer the ozone concentrations used caused lenticel injury to the fruit.

Discussion: Earlier investigators were not able to control scald on apples, but they used amounts of ozone so small that they could not analyze for it. The experiments reported above suggest there may be possibilities in scald control when high concentrations of ozone are used. It should be noted however in the experiments described that nearly ideal mixing and air movement was had around the fruit and this condition might be hard to obtain under commercial conditions.

2.4.2 Smock and Watson (1941) – Apple Storage Rooms

Abstract: In the interesting series of tests which the authors describe here, the use of ozone was found to reduce materially the mold spore count in apple storage rooms. It was also found to check the spread of rots on scabby apples significantly. The effect of ozone on reducing the ripening rate of apples is not clear cut, the authors say, but is in favor of the ozone treatment. They point out that more study is needed on the effect of ozone on apple scald in storage. Mr. Smock and his associates have done extensive work for years in "controlled atmosphere storage" of apples, a method which is gaining popularity among growers in New York and Now England.

This report of progress concerns itself chiefly with the problem of fungi in the apple storage although some other aspects of ozone in this type of storage are discussed. Various fungi are a source of trouble in the apple storage on at least three counts. First, they are a source of rotting of fruit. Second, they grow as surface molds on the walls, containers, and sometimes on the fruits themselves. Third, they contribute to musty or foul odors in the storage. These odors are often absorbed by the flesh of the fruits and decrease their sales value.

The source of ozone in laboratory experiments was from small units containing just one glass dielectric or from quartz lamps. Ewell (1940) has recently shown that ozone from either of these sources is apparently identical (**Far from the Case Today** as far as **quantity** of output is concerned. On the other hand, ozone **IS** ozone, regardless of how it is generated). Experiments on large lots of fruit were conducted with commercial models of ozone generators. Ozone was measured by the usual sodium thiosulfate-iodine titration or by special ambient air ozone monitors today.

Surface molds are likely to be a problem in the apple storage when the relative humidity is 80 percent or above. The standard recommendation in the past has been 80 to 85 percent for the apple room. This is merely a compromise figure between that humidity which will prevent shriveling and that which will inhibit mold growth. From the standpoint of apple shriveling, the relative humidity of the room should be 95 percent or more. In some varieties, visible shriveling and loss of crispness occur in long time storage below 95 percent relative humidity. As has been indicated, these high humidities have been impossible at times because of excessive mold growths. Another reason for the desirability of using high humidities in apple storage is that

fruits which are very susceptible to the trouble known as "bitter pit" or “stippin" do not develop this disorder as quickly in storage in very high humidities as in low.

Effect of Ozone on Spore Germination:

This experiment was designed to test the effect of ozone on the germination of spores of three different fungi. Slices of apple were floated on water in petri dishes and the spores placed on the skin. Spore germination tests were made by counting spores removed with celloidin films. Table 2.4.1 reveals that continued treatment with ozone definitely inhibits the rate of germination of these three fungi (*Sclerotinia fructicola, Penicillium expansum, Macrosporium*) . *Macrosporium* was the least affected, probably because it is multi-celled and larger than the other two. At the end of two hours plasmolysis was noted in the spores of *P. expansum and S. fructicola*. It seemed that 0.6 ppm of ozone would kill spores in 3 to 4 hr. if the spores were not in clumps. Dry spores seemed to kill nearly as rapidly as wet spores at room temperature in the presence of this concentration of ozone.

Table 2.4.1. Effect of 0.6 ppm Ozone on Germination of Wet Spores of Three Fungi at Room Temperature (Smock and Watson, 1941)

Fungi	**Ozone, Treatment hours**						**No ozone, hrs**		
	0.5	**1**	**1.5**	**2.**	**2.5**	**3**	**0.5**	**4**	**5.5**
	% spores remaining								
S. fructicola	61	23	18	16	0	0	70	57	60
P. expansum	....	69	50	3	0	0	68	65	70
Macrosporium	98	97	96	40	15	6	100	97	96

Spore Counts in Storage Room Air: Spores were collected on agar plates for one to five minutes exposure in three different storages on different dates. In only one of the storages was the ozone concentration measured frequently and maintained at a given concentration more or less regularly. In storage 2 (a room that had a very serious mold problem before the use of ozone was started), insufficient ozone was used to be measurable by the Ewell method. The relative humidity was from 85 to 90 percent in all of these rooms.

Table 2.4.2 indicates that continuous ozone operation lowered the spore count strikingly. Practical results judged from observable hyphae on the boxes and the fruit at the end of the storage season indicated that one or two hours operation a day is sufficient. For best results, however, one or two parts per million should be used each day for one hour or two. Storage 2 was a room that had a very serious mold problem before the use of ozone was started. The mold spore count was very high in this room before the initiation of the ozone trial. Not enough ozone was used to reduce this spore count very strikingly. Traces of surface mold could be seen growing on the storage containers at the end of the storage season in this room. Where the spore count was as low as it was in storage 3, no surface molds could be seen on the containers at the end of the season. Of course, spores may have been present and yet no visible mold growth, but it would seem from laboratory trials that ozone both inhibits mold growth and kills spores.

Table 2.4.2. Spore Counts in Ozone and Non-ozone Cold Storage Rooms for Apples (Smock and Watson, 1941)

Storage Number	Temperature EF	Date	Ozone Conc'n, ppm	Total spores/yd^3 atmosphere	
				Ozone	No Ozone
1	40	11/22/40	1.5*	16,000	1,380,000
1	40	12/26/40	1.5*	2,760	4,280,000
2	32	2/26/41	....**	910,000	1,111,000
3	32	11/18/40	0.4***	46,000	.....
3	32	12/21/40	0.4	81,000	.....

* Ozone was used continuously in this room.

** Ozone was turned off when concentration could be smelled.

*** Ozone was used only for an hour or two each day.

Effect of Ozone on Rotting of Apples:

It would seem that ozone is certainly not a means of control of rotting of apples in storage. In certain cases, however, it may inhibit the amount of rotting. Ozone prevents aerial growths of the fungus and undoubtedly prevents considerable spreading of spores. It certainly cannot prevent rotting of inoculated apples, however. On moist, exposed surfaces of apple flesh the spores are protected from the ozone.

Effect of Ozone on Odors in Apple Storage:

Often surface molds produce a musty, foul odor. Molds and bacteria growing in abundance together on moist surfaces often produce rancid, vile odors. Two demonstration experiments were conducted in which the sources of odors were molds or combinations of molds and bacteria growing on the moist surfaces of the storage room. In both cases the apples under examination were Northern Spy. This variety is often troublesome in this regard since it seems to absorb odors more quickly than some other sorts.

In one storage which had an odor problem, 2-4 parts per million of ozone were used for several hours each day. The absorption of odors by the fruit was eliminated by, this procedure. In the second storage an unmeasured amount of ozone was used daily after the odor problem was apparent. The odor was a very rancid one and isolations seemed to indicate that a large number of fungi and bacteria were involved. Although the amount of ozone was so low it could not be measured (it could be smelled) the odor problem was greatly alleviated in this storage even after it had become rather severe.

Effect of Ozone on Ripening of Apples:

The effect of ozone on the general ripening rate was tested further in one experiment with Rhode Island Greening apples stored with several rapidly ripening varieties. The experiment was conducted in semi -commercial rooms of about 800 bushels capacity each. One to two parts of

ozone were maintained throughout the treated room continuously during the entire experiment. The temperature of both rooms was 40EF. With this continuous application of ozone the Greenings in the treated room became slightly sticky. Something happened to the natural wax on the fruit, which made it bright and slightly adhesive to the touch. Any effect of the treatment on ripening might have been due to this effect on the natural wax. That is, if the wax was made less permeable to gases the ripening rate may have been slowed up for this reason and not because of any direct effect on ethylene or other factor.

Conclusions: ***In the experiments herein described, the use of 1-2 parts per million of ozone used an hour or two each day in the apple storage controlled the growth of surface molds.***

The use of ozone was found to reduce materially the mold spore count in apple storage rooms.

Careful handling of the fruit is the most important consideration in rot control. The use of ozone while not a control is a step in the right direction from the standpoint of apple rots. It checked the spread of rots on scabby apples significantly. Ozone prevents any aerial growth of the rotting fungus and may prevent spreading of spores in this way. Ozone definitely checks the rate of spore germination and actually plasmolyzes mold spores. If the spores are protected by moist surfaces of apple flesh or by other organic protectants, the ozone may have no effect on germination.

The effect of ozone on reducing the ripening rate of apples is not clear-cut, but is in favor of ozone treatment. Ozone although a powerful oxidizing agent does not increase the respiration rate of apples and there is some indication that it may nullify the ripening effect that an early variety of apples may have on another variety. This effect is not of great commercial importance, however. The use of ozone may be expected to remove some of the offensive odors that accumulate in an apple storage room.

The effect of ozone on apple scald in storage is not clear-cut. More study is needed on this point. While ozone may in no wise be considered a control of apple scald, there is a little evidence indicating that it may reduce the intensity of scald if used in sufficient concentrations.

2.4.3 Kuprianoff (1953) – Fruit Storage Conditions

The general effects of ozone important under fruit storage conditions may be summarized as follows

Bactericidal Effect: Ozone is a weaker bactericide in air than fungicide. It must be present in a relatively high concentration to be effective against bacteria. At the low concentration of 0.2 mg O_3/m^3 air, it encourages rather than discourages bacterial growth. The relative humidity of air is a very important factor. Another problem is that bacteria can acclimate to ozone after long treatments.

Fungicidal Effect: Only small concentrations of ozone are needed to prevent the growth of fungi on mold and fungus free surfaces. However, somewhat higher ozone concentration is needed for the destruction of existing colonies. Thus the single cells on the surface of the fruit are more readily destroyed. The reason for it is that the ozone cannot penetrate deeply into the fruit. With increasing humidity, the fungicidal effect increases. This observation indicates that

the moisture content of the surface is important inasmuch as the ozone can attack the fungus only when it is in a swollen state. Yeasts are more readily attacked by ozone than bacteria and the spores of mildew fungus are even more resistant to ozone than are bacteria.

Sterilization of Air: The sterilization of air by ozone can be achieved only with difficulty. The humidity of the air must be at an optimum value. High humidity is required since the airborne microorganisms must be in a swollen state especially since they are usually present in the air in their spore form. On the other hand, ozone decomposes more readily at high humidity. The ozone must be thoroughly and evenly distributed. As a result of the rapid decomposition, no ozone remains for long in the storage area under these conditions when the ozone feed is discontinued. This is an important observation for workmen who need to enter the storage room.

Effect on Microorganisms on the Fruit: Ozone does not penetrate deeply, only acts on the surface. Therefore, the fruit must be packaged to allow a free circulation of ozone-containing air, at least under forced circulation conditions. The concentration of ozone must be sufficiently high to allow for its decomposition on the walls of the storage room, on the crates and on the other objects present, and still remain in sufficient concentration for the bactericidal and fungicidal action.

Effect on Storage Room and Equipment: Ozone controls the mildew formation on the storage room walls and storage crates, and other packaging materials. The blue mildew can grow well if not checked by ozone under the cold storage room conditions at 0EC and impart a mildewy taste and odor to the fruit. Ozone treatment allows the disinfection and deodorization of the storage room between changes of fruit to be stored. However, the odor control effect is limited to oxidizable odorous materials. The rotting smell is not removed. The lower temperature slows the odor control, but relative humidity does not affect it. A low 0.01-0.04 cm^3 O_3/m^3 air eliminates mildewy odor and imparts a fresh smell to the air.

Continuous ozonation of cold storage room air effectively controls the odor when it is used in combination with a central air cooling system or separate cooling units for each storage area. Further, it prevents the circulation of odors from one area to the other. In aromatic fruits (e.g., strawberries) ozone strengthens the aromatic flavor, possibly because in the presence of ozone the fruit produces more aroma. The storage boxes develop an odor in the 80-90% relative humidity of the storage room. The ozone treatment controls these odors very effectively.

Materials of Construction: In an ozone atmosphere of 25-40 mg O_3/m^3 metals are oxidized and must be protected by painting. However, under the normal ozone concentration used in fruit storage (5 mg O_3/m^3 or less) the corrosion effect is minimal and without much consequence. The ducts and ozone piping should be made of aluminum or stainless steel. The sealing materials on doors and windows should be made of ozone-resistant synthetic materials.

Effectiveness of Ozone on Fruits: In order to be effective the ozone must be present in the air above the fruit and on the surface of the fruit. It cannot penetrate deep into the fruit. The effect of ozone is dependent on the following parameters:

Ozone Concentration is the most important parameter. It was difficult to measure in the past due to the unavailability of good detectors or analytical methods and due to the rapid

decomposition of ozone and varying reactivity on different materials and surfaces. Much of the discrepancy of the earlier results using ozone treatment for fruit storage may be explained by inaccurate measurement of ozone concentration and the uneven distribution of ozone in the air. The most frequently used concentration is 1-3 cm^3 O_3/m^3 air in the cold storage room for fruit.

Treatment Time can be limited to a few hours several times a day. The even distribution of ozone in the storage room must be assured by good circulation and mixing of the air. The fruit storage 2-3 cm^3 O_3 $/m^3$ air is recommended for 2-3 hours two to three times daily. A much higher concentration (e.g., 400 mg/m^3 does not improve the fungicidal effect in most cases.

Relative Humidity influences the ozone decomposition rate and the swelling of the microorganism. At a 100% relative humidity, ozone decomposes very rapidly much faster than at low humidity. The dehydrated microorganism is relatively resistant to ozone. It has to take up water and be in a swollen state to be more readily susceptible to ozone. By the use of ozone, it is possible to increase the relative humidity safely and to avoid weight loss in the fruit through dehydration.

Under the condition of relative humidity 50% or less, ozone loses its bactericidal effect. The optimum relative humidity is in the range of 90-95% for ozone treatment. It is fortunate that in fruit cold storage rooms, they normally use relative humidity of 85-95%. Under these conditions, ozone effectively controls surface microorganism growth with no weight loss to the fruit. Under these conditions, the shrinkage of the apples is also eliminated.

Temperature has a strong effect also. The ozone treatment of food materials loses its germicidal effect above 10EC, when the usual concentrations are used. The ozone inhibition of microorganism growth above this temperature is no longer sufficient. On the other hand below this temperature, the metabolism of the microorganism is so slow that ozone treatment can overwhelm it. Therefore, at lower temperature the required ozone treatment time becomes shorter. It must be taken into account that at lower temperature, the ozone generators produce more ozone to avoid excessive ozone concentrations.

2.4.4 Spotts and Cervantes (1992) – Spores and Pear Fruit

Abstract: In treatments with aqueous ozone solutions for 5 min, LD_{95} values for spores of *Botrytis cinerea*, *Mucor piriformis*, and *Penicillium expansum* were calculated to be 0.99, 0.69, and 0.39 Φg of ozone per milliliter of water, respectively. Spore inhibition was directly correlated with ozone concentration in 1 to 5-min exposure times; eight regressions of spore inhibition with concentration were significant at P = 0.01, and one was significant at P = 0.05. However, in pear fruit (*Pyrus communis* d'Anjou) wound-inoculated with *P. expansum* and then treated with water containing up to 5.5 Φg of ozone per milliliter for 5 min, levels of decay were similar to those of a control treated with water alone. In a commercial packinghouse test, fewer propagules of *Altenaria spp*. were recovered from chlorinated water than from ozonated water, whereas equal numbers of propagules of both *Cladosporium* and *Penicillium spp.* were recovered. Similar levels of decay were recorded in pear fruit floated through ozonated dump tank water and in fruit treated with chlorinated water, after storage at -1EC for 5 months.

The objectives of this study were to determine the toxicity of aqueous ozone solutions to spores of *R cinerea, M. piriformis*, and *P. expansum*; to determine the effectiveness of ozonated water in the control of disease in pear fruit wound inoculated with spores of these fungi and to compare ozonated with chlorinated dump tank water in a commercial packinghouse with respect to the control of fungal populations in flotation tanks and their effects on pear decay.

Materials and Methods

Effect of Ozone on Spore Germination: Ozone was produced by passing high purity oxygen through a corona discharge ozone generator. The ozone was mixed with circulating water at 10EC to produce concentrations of 0.1 - 4.0 Φg of ozone per milliliter. The pH of the solutions was 7.5 and was not affected by the ozone concentration. The concentrations were measured with the neutral buffered potassium iodide method. Spores of *B. cinerea, M. piriformis*, and *P. expansum* were washed from 7-day-old plates of acidified potato dextrose agar (APDA, acidified with 1.5 mL of 85% lactic acid per liter) and suspended in sterile distilled water. This stock suspension was added to the ozonated water to produce a final concentration of 4 x 10^3 spores per milliliter at the stated ozone concentration. After exposure times of 0 (control), 1, 3, and 5 min, a 10-mL sample was removed, the liquid phase was removed with a 0.45-Φm Millipore filter, and the spores retained on the filter were washed with 5 mL of sterile distilled water. Next, the spores were blotted onto the surface of an APDA plate. Plates with spores of *B. cinerea* and *M. piriformis* were incubated at 10EC, and those with *P. expansum* at 15EC for 24 hr. Germination of 100 spores was examined microscopically at 400 X. A spore was considered germinated if the length of the germ tube was at least half the diameter of the spore. The experiment was done three times for each fungus. The percentage of inhibition of germination caused by the ozone was calculated from the germination of spores in water. Data were analyzed by regression of $\log_{10}$ ozone concentration; with the ozone concentrations averaged over the exposure intervals.

Effects of Ozone and Chlorine on Concentration of Fungal Propagules in Dump Water and on Pear Decay in a Commercial Packinghouse. D'Anjou pear fruit from three grower lots were divided into two subgroups per lot. One sub-group from each lot was floated through a dump tank containing 18,900 L of an aqueous solution of sodium silicate (used as a flotation agent) in which sodium hypochlorite was dissolved. The sodium hypochlorite concentration was adjusted so that the total available chlorine was 54 ∀ 16 Φg/mL, as determined by sodium thiosulfate titration. The specific gravity of the sodium silicate solution was 1.026, the pH was 11.6, and the temperature was 5EC. Individual fruit were in the tank about 5 min. These sub-groups were processed on 24 and 25 October 1990. On 31 October and 1 November 1990, the remaining sub-groups were processed in the same sequential order, but sodium hypochlorite was replaced with ozone produced by a commercial generator at 0.31 ∀ 0.09 Φg/mL. The pH of the solution was 11.2. Ozone concentrations were determined by an ozone test kit based on the indigo method (Hach Co., Loveland, CO). The water was sampled three times each day at 2-hr intervals from three locations in the tank. The samples were diluted 1:99 with sterile distilled water, and 0.2-mL aliquots were spread over the surface of five APDA Petri plates per sample. Fungi that developed in the plates were identified and enumerated after 5 days of incubation at 22EC. At each of the sampling times, about 40 kg of fruit was removed from the cull bin, enough to fill two polyethylene-lined cardboard boxes. Most of these cull fruit contained natural

punctures and were culled after the dump tank but prior to a fungicide line spray. One box of each pair was stored at -1EC and examined monthly for 5 months. The second box was held at 20EC and examined at 7 and 14 days. At each examination, decay incidence was assessed and categorized on the basis of visual symptoms.

Results

Effect of Ozone on Spore Germination: The relationship between the $\log_{10}$ ozone concentration in water and the $\log_{10}$ percent inhibition of spore germination was linear for all three fungi (Table 2.4.3 and Figure 2.4.1). As determined by these regressions, the LD_{95} for all fungi was an ozone concentration of less than 1 Φg/mL in a 5-min exposure or between about 1.5 and 2.5 Φg/mL in a 1-min exposure. Similarly, if spores were exposed to ozone at 0.1 and 0.5 Φg/mL for 1 min, generally less than 50% of germination was inhibited, whereas with a 5-min exposure, 83-96% was inhibited. *M. piriformis* and *P. expansum* were similarly sensitive to ozone, but *B. cinerea* was more sensitive than these fungi to ozone concentrations less than about 1.0 Φg/mL for up to 3 min. At higher ozone concentrations or longer exposure times (5 min), *B. cinerea* was less sensitive than the other fungi (Figure 2.4.1).

Effects of Ozone and Chlorine on Concentration of Fungal Propagules in Dump Water and on Pear Decay in a Commercial Packinghouse: The three most common fungi isolated from the dump tank water were *Alternaria spp*., *Cladosporium spp.*, and *Penicillium spp.* No consistent differences were observed in fungal populations in the three sample locations in the tank, and the values were averaged at each sampling time. The number of viable propagules of *Alternaria spp*. in chlorinated water in the packinghouse dump tanks was about one-tenth the number isolated from ozonated water (50 vs. 488 cfu/mL). In contrast, the numbers of propagules of *Cladosporium spp.* (1,317 and 2,308 cfu/mL) and *Penicillium spp*. (305 and 439 cfu/mL) in the two treatments were not significantly different at P = 0.05.

Table 2.4.3. Regression equations for concentration of ozone in distilled water and inhibition of spore germination of three fungi pathogenic on pear fruit (Spotts and Cervantes, 1992)

Fungus	Exposure time, min	Regression equation[a]	Correlation coefficient[b]
Botrytis cinerea	1	log Y = 1.843 + 0.344 log X	0.869
	3	log Y = 1.955 + 0.094 log X	0.888
	5	log Y = 1.978 + 0.061 log X	0.850
Mucor piriformis	1	log Y = 1.815 + 0.815 log X	0.924
	3	log Y = 1.972 + 0.231 log X	0.895
	5	log Y= 1.987 + 0.058 log X	0.867
Penicillium expansum	1	log Y = 1.822 + 0.7 18 log X	0.813
	3	log Y = 1.971 + 0.233 log X	0.893
	5	log Y = 2.000 + 0.051 log X	0.785

[a] Y = percent inhibition of germination; X = Φg of ozone per milliliter of distilled water.
[b] Significant at P = 0.01 (except for *P. expansum* in the 5-min exposure; significant at P = 0.05).

Five different decays were identified among the fruit from the chlorine or ozone water treatments (Table 2.4.4). After 14 days at 20EC, the decays caused by *M. piriformis* and *Penicillium spp.*) were the most common. Total decay and decay caused by *Penicillium spp*. were less in the chlorine treatment than in the ozone treatment. In 5 months of storage at -1EC, the most common diseases in fruit were caused by *Alternaria spp.* and *Penicillium spp*. No differences in disease incidence were observed between fruit from the chlorine treatment and fruit from the ozone treatment at this storage temperature.

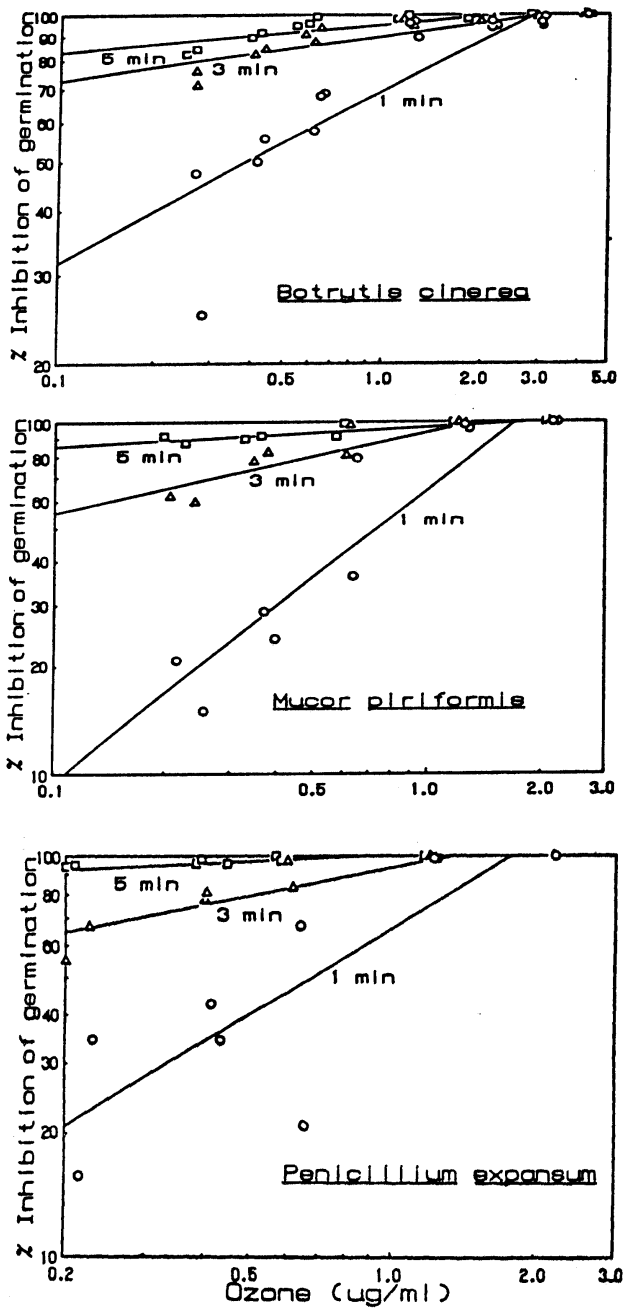


Figure 2.4.1. Inhibition of spore germination of three decay fungi after treatment with 1-, 3-, and 5-min exposures to ozone in various concentrations in water. Regression equations for all lines are given in Table 2.4.3 (Spotts and Cervantes, 1992).

Table 2.4.4. Decay of d'Anjou pear fruit after ozonated and chlorinated water treatments in a commercial packinghouse (Spotts and Cervantes, 1992)

Pathogen	Percentage of fruit-decayed[a] at storage temperature of:			
	20EC[b]		-1EC[c]	
	Chlorine[d]	Ozone[e]	Chlorine	Ozone
Alternaria spp.	0.3	0.7	7.1	6.3
Botrytis spp.	0.4	0.3	3.1	3.0
Mucor piriformis[f]	10.0	9.5	3.9	2.0
Penicillium spp	7.8*	20.8	5.1	11.0
Pezicula malicorticis	0.0	0.0	1.6	0.8
Total decay	18.5**	31.3	20.8	23.3

[a] Significant differences between chlorine and ozone treatments according to the unpaired t test at P = 0.05 and 0.01 are indicated by * and **, respectively. Each value is based on six boxes of d'Anjou pear fruit, each box containing about 20 kg.

[b] Evaluated after 7 and 17 days.

[c] Evaluated monthly for 5 months.

[d] Sodium hypochlorite in sodium silicate flotation solution, with total available chlorine adjusted to 54 ∀ 16 Φg/mL, pH 11.6, at 5EC.

[e] Ozone in sodium silicate flotation solution, with ozone concentration adjusted to 0.31 ∀ 0.09 Φg/mL pH 11.2 at 5EC.

[f] includes fruit infected with *Rhizopus stolonifer* at 20EC.

Discussion: The germination of spores of three common decay fungi was inhibited by treatment with ozonated water. However, from our results and those of previous studies, the concentration of ozone required to kill fungal spores is considerably higher than the levels used to inactivate bacteria, viruses, and *Giardia* cysts. For example, we calculated that the LD_{95} for conidia of *B. cinerea* with a 5-min exposure was 0.99 Φg/mL. In contrast, the LD_{99} for *E. coli* with a 0.33-min exposure is only 0.06 Φg/mL.

Ozonated water did not control decay in wound-inoculated pear fruit. Ozone in air does not reduce infection in inoculated wounds in apple. Also, ozonated water fails to inactivate spores of *B. cinerea* placed in surface injuries of tomato fruit. Similarly, a lack of decay control has been observed in inoculated fruit treated with chlorine. Apparently, strong oxidants such as ozone and chlorine react with plant tissue and extracellular biochemicals at wound sites and often fail to inactivate microbes attached to or embedded in plant tissue. Both chlorine and ozone -react rapidly with organic matter. The activity of both chlorine and ozone is affected by the pH of the solution. Chlorine is more effective for decay control in a sodium sulfate solution at pH 7.8 than in sodium silicate at pH 11.2. Similar information -relating the pH of ozone solutions to decay control is not available.

At the commercial storage temperature of -1EC, similar decay levels developed in fruit floated in chlorinated dump solutions and fruit in ozonated dump solutions in a packinghouse. We were

not permitted to include a water control in the commercial packinghouse trials and had to compare ozone with chlorine as the industry standard. We have shown in this study that ozone inhibits the germination of spores of *B. cinerea*, *M. piriformis*, and *P. expansum*. If a similar effect occurred in dump tank water, we would expect an indirect mode of decay control to occur as the concentration of viable fungal propagules was lowered. A positive relationship has been established between decay of d'Anjou pear fruit and the concentration of viable spores of *B. cinerea*, *M. piriformis*, and *P. expansum* in water.

For spores of *B. cinerea*, *M. piriformis*, and *P. expansum* the LD_{99-100} of chlorine at pH 9 in a 5-min exposure was about 50 Φg/mL. To achieve this level of inhibition of these fungi with ozone solutions in 5 min, concentrations of 1.9, 1.4, and 0.8 Φg/mL, respectively, were required. These are considerably higher concentrations than the 0.31 Φg/mL used in our packinghouse tests and may explain the higher recovery of fungal propagules from dump tank water treated with ozone rather than chlorine. After one test, the ozone concentration was increased, and slight off-gassing occurred. Additional research is needed to determine the maximum amount of ozone that can be dissolved safely in packinghouse dump tank water and to relate that concentration to efficacy against the accumulation of spores of fungal pathogens in the water.

2.4.5 Barth et al. (1995) – Thornless Blackberries

Abstract: Ozone exposure was assessed for storage of thornless blackberries, which are prone to fungal decay. Blackberries were harvested and stored for 12 days at 2EC in 0.0, 0.1, and 0.3 ppm ozone. Berries were evaluated for fungal decay, anthocyanins, color and peroxidase (POD) activity. Ozone suppressed fungal development for 12 days, while 20% of control fruits showed decay. The main mold was *Botrytis cinerea*. Ozone storage did not cause observable injury or defects. By 12 days, anthocyanin content of juice was similar to initial levels for all treatments. Surface color was better retained in 0.1 and 0.3 ppm-stored berries by 5 days and in 0.3 ppm berries by 12 days, by hue angle values. POD was greater in controls and 0.1 ppm samples, and was lowest in 0.3 ppm fruits by 12 days. Ozone storage resulted in market quality extension.

Materials and Methods

Fruit and Storage: Blackberry fruit (cv Chester) was produced in field plots at the Univ. of Kentucky. Fruit was harvested, transported to the Univ. of Kentucky Agricultural Engineering Pilot Plant, handled according to commercial practice, weighed into perforated clam shell containers (140 g) and placed in three cold rooms controlled at 2EC, 90% RH and ozone concentrations of 0.0, 0.1, 0.3 ppm.. Temperature, relative humidity and ozone concentration of each cold room was computer-controlled. The temperature was controlled by the refrigeration system ∀ 1.2EC. A sensor was used to measure relative humidity and was checked using a chart hygrometer. Cold room temperatures were confirmed with thermometers and by the temperature measurement from the hygrometer. Ozone was produced using ozone generators (Model PA600, Zontec Inc., Ogdensburg,, NY). Ozone concentration was measured using a Dasibi UV detector which had been factory calibrated. The relative humidity arid ozone concentration in each cold room were measured and controlled using OPTOMOX, analog and digital data acquisition board. A computer program using Professional Basic was written to record the analog data and engage the ozone generator, electric heater and humidifier. Accuracies were ∀ 5% for relative humidity, and ∀ 0.01 ppm for ozone concentration.

At each sampling interval (0, 1, 2, 5, and 12 days) duplicate containers were removed for analysis of fungal growth, total anthocyanin content, color, and moisture. POD activity was measured at 0, 2, 5. and 12 days of storage. Each experiment was repeated two times and an analysis of variance was conducted on the data.

Estimation of Fungal Decay: Fungal infection was estimated visually during the course of each experiment. Blackberry fruit showing surface mycelial development were considered decayed. Fungal infection was expressed as percent of berries infected per container. Fungal pathogens were isolated by plating infected berries on potato dextrose agar supplemented with 100 ppm chloramphenicol and identified by visual characteristics.

Results

Estimation of Fungal Decay: Fungal decay was not observed in the ozone-treated fruit by 12 days. In contrast, 20% of controls (0.0 ppm) showed visible signs of fungal growth and decay by day 12. *B. cinerea* was the main cause of infection, although *Rhizopus sp.* caused some decay in the scar area of the fruits.

Discussion

Storage of Blackberry Fruit under continuous ozone of 0.1 or 0.3 ppm was very effective in preventing fungal decay for up to 12 days at 2EC. Ozonated water has been shown to inactivate spores of *B. cinerea* (Spotts and Cervantes, 1992). However, short exposure to ozone was not effective in killing spores of *B. cinerea* and other fungi at the wounded surface of tomato and pear fruits (Spotts and Cervantes, 1992). Likewise, a short prestorage exposure to ozone was not effective in preventing decay of pear fruit (Spotts and Cervantes, 1992). It thus appears that constant exposure to ozone throughout storage is more effective in inhibiting storage pathogens as shown here (0.1 and 0.3 pprn, 2EC, 90% RH).

Ozone as a storage treatment is a likely alternate to use of fungicides for control of post-harvest rot in small fruit. Our computerized control system for ozone storage allowed for maintenance of more constant levels of ozone throughout storage as compared with earlier studies. Ozone storage was beneficial for quality preservation of blackberries and may be effective for quality preservation of other small fruit.

2.4.6 Sarig et al. (1996) – Grapes

Abstract: The effect of ozone on post-harvest decay of table grapes was studied both with regard to its effectiveness and its possible mode of action. Ozone concentrations fell rapidly upon contact with organic matter and the amount which reacted with grape berries and the microflora on their surface was about 0.1 mg g^{-1} when supplied at a rate of 8 mg min^{-1} for 20 min. The dose applied could be increased by longer periods of exposure, but symptoms of toxicity were observed on certain cultivars. The number of colony forming units (cfu) of fungi, yeasts and bacteria naturally present on the berry surface was considerably reduced by a 20 min exposure to ozone. Ozone treatments significantly reduced the extent of berry decay caused by fungi following cold storage and increased shelf-life. A significant decrease in decay was observed in berries that were treated with ozone either before or after being inoculated with

Rhizopus stolonifer. This finding indicates that, in addition to its sterilizing effect, ozone also induced resistance to post-harvest decay development. The phytoalexins resveratrol and pterostilbene were elicited by ozone treatments, at levels similar to those produced by UV-c irradiation. Resveratrol accumulated in greater quantities than pterostilbene. Inoculation with *R. stolonifer* in addition to ozone treatment raised the levels of both stilbenes even more. Exposing berries to ozone was almost as effective as SO_2 fumigation for the control of storage decay caused by *R. stolonifer* and no deleterious effects were observed on the appearance of the grape cluster. Ozone treatments therefore can be considered as a possible substitute for SO_2 fumigation for the control of post-harvest fungal decay.

In the present work, we examined the effects of exposing grapes to ozone on the control of post-harvest control of fungal decay, and present evidence to show that this is achieved as a result of both surface sterilization and elicitation of stilbene phytoalexins.

Materials and Methods

Plant Material: The table grapes (*Vitis vinifera L.)* used throughout these studies were harvested from commercial vineyards in Israel and brought to the laboratory immediately after harvest. Cultivar Perlette was harvested in July 1993, cvs Thompson Seedless and Zeiny in September 1993, and cvs Alphonse Lavallée and Barlinka in November 1993.

Fungal Inoculations: *Rhizopus stolonifer* was isolated from diseased berries, and established as a single spore culture on potato dextrose agar (PDA), and maintained at 4EC. To prepare spore suspensions, the fungus was grown for 3 days in two Petri dishes containing PDA. The spores were gently removed from the cultures with 10 mL sterile distilled water per dish. The suspension was transferred to a sterile flask and the final spore concentration was adjusted to 10^7 mL^{-1}. (This high spore concentration was used to ensure infection of the fruit without wounding). The grapes then were inoculated by immersing berries detached from clusters with their pedicels, in the suspension for 1 min. After inoculation, each berry was placed in all isolated well of a sterile polystyrene plate and incubated at 28EC in a saturated atmosphere. The percentage of infected berries was recorded after 72 h.

Ozone Treatments in Small-Scale Trials: Approximately 2 kg of grape clusters of each cultivar were exposed to ozone for different periods of time (up to 30 min), in a 30-L screw-capped Plexiglas cylinder, adapted with an entrance port for ozone application and an exit port for air exclusion. Throughout the experiment, the sealed cylinder was held on a laminar flow bench to minimize environmental contamination. Ozone was supplied at a rate of 8 mg min^{-1} in an air flow of 500 mL min^{-1}. The amount of ozone supplied was monitored with the aid of a spectrophotometer adjusted to 254 nm and fed with a bypass pipe from the entrance of the cylinder. The readings were calibrated by comparison with the values obtained by iodometric titration. The values obtained were constant throughout the experiment. The air expelled from the cylinder was captured in a flask containing 400 mL of 20 g L^{-1} KI solution and the amount of trapped ozone was determined by the iodometric titration method. The difference between the levels of ingressing and egressing ozone was calculated and defined as the amount that had reacted with the grapes and with the microflora on the grape surface. Control measurements, without fruit, indicated that the amount of spontaneous ozone degradation was negligible under

the experimental conditions. After treatments, clusters were transferred to sealed polyethylene bags and stored as described below.

Effect of Ozone on the Microflora Present on Berry Surfaces: The effect of ozone treatment on fungi, yeasts and bacteria (as cfu) was determined after different periods of time. Following ozone treatment, five berries were detached from different clusters, immersed in sterile distilled water and shaken for 30 min. For each treatment, three 20 ΦL samples were sampled from the aqueous suspensions. Each sample was spread over selective media. The inoculated Petri dishes were incubated at 28EC for 24 h and the number of colonies was recorded.

Effect of Ozone on Grape Decay, with or Without Inoculation with *Rhizopus stolonifer:* Grapes were treated with ozone and inoculated with R. stolonifer as described above. Four categories of treatment were used: (*i*) clusters brought to the laboratory immediately after harvest were submitted to ozone treatment for different periods of' time (up to 80 min); (*ii*) as in (*i*), but ozone was applied after immersing the clusters in 70% ethyl alcohol; (*iii*) as in (*i*), but before ozone treatment the clusters were inoculated by immersion in a *R. stolonifer* spore suspension of 10^9 mL^{-1}; and (*iv*) as in (*i*), followed by inoculation as in (*iii*). After treatment, grape clusters were incubated at 20EC and 85 90% RH. Extractions for phytoalexin determination were performed 24 h after the termination of ozone treatments. The number of berries showing decay was recorded after 6 days' incubation.

Results

Ozone Degradation During Exposure of Grapes: Increasing ozone doses were achieved by extending the exposure time. The amount of ozone reacting with the grapes and the microorganisms on their surface was approximately 0.1 mg g^{-1} grapes during 20 min of exposure. This level was maintained with little change for an additional 20 min. Increasing the exposure time up to 80 min caused a linear increase in reacted ozone.

Effect of Ozone on the Microflora Naturally Present on Berry Surfaces: Ozone significantly reduced the microfloral population on berries of all three cultivars after exposure for 1 min only (Figure 2.4.2). Total elimination of all three groups (fungi, yeasts and bacteria) was achieved by 20 and 40 min exposure of cvs. - Alphonse Lavallée and Thompson Seedless, respectively. The microflora on cv. - Zeiny, especially the bacteria, were less affected by ozone treatment. Immersing berries in 70% ethanol generally was as effective in reducing the microflora levels as a 5 min exposure to ozone, with the exception of yeasts and bacteria in cv. Zeiny. In the latter cases, a combination of' ethanol immersion and 20 min exposure to ozone almost eliminated the whole microfloral population.

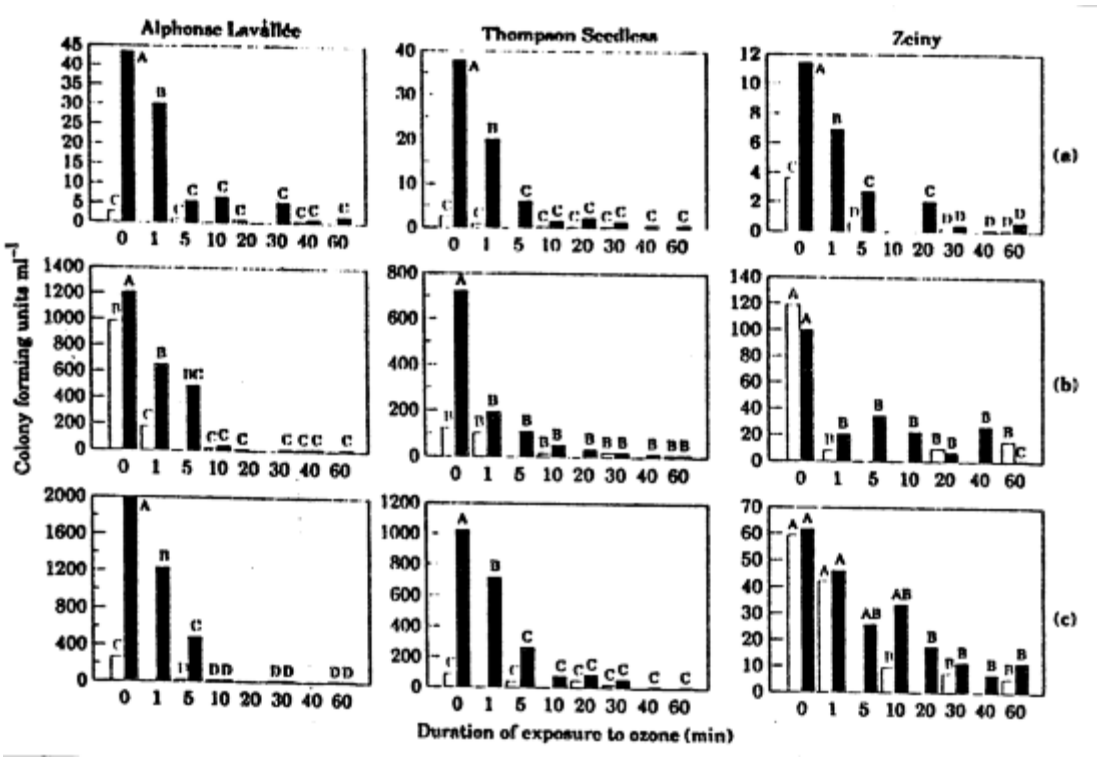


Figure 2.4.2. Colony forming units of (a) fungi, (b) yeasts and (c) bacteria on berries of cvs. Alphonse Lavellée, Thompson Seedless and Zeiny after exposure to ozone for different periods of time. Berries were immersed (G) or not (#) in 70% ethanol prior to ozone treatments (assessments made after ozone exposure). Columns with different letters in each figure indicate significant differences by Duncan's multiple range test (P # 0.05) (Sarig et al., 1996).

Effects of Ozone on Berry Decay after Storage and Simulated Shelf-Life: In three of the four treatments described in Materials and Methods, exposure to ozone significantly decreased the number of berries susceptible to the fungi (Figure 2.4.3). On fruit immersed in ethanol, no decay developed during 6 days at 20EC, even without exposure to ozone. Thirty to forty minutes of exposure was sufficient to eliminate all decay, even in treatments where berries were heavily inoculated with *Rhizopus*.

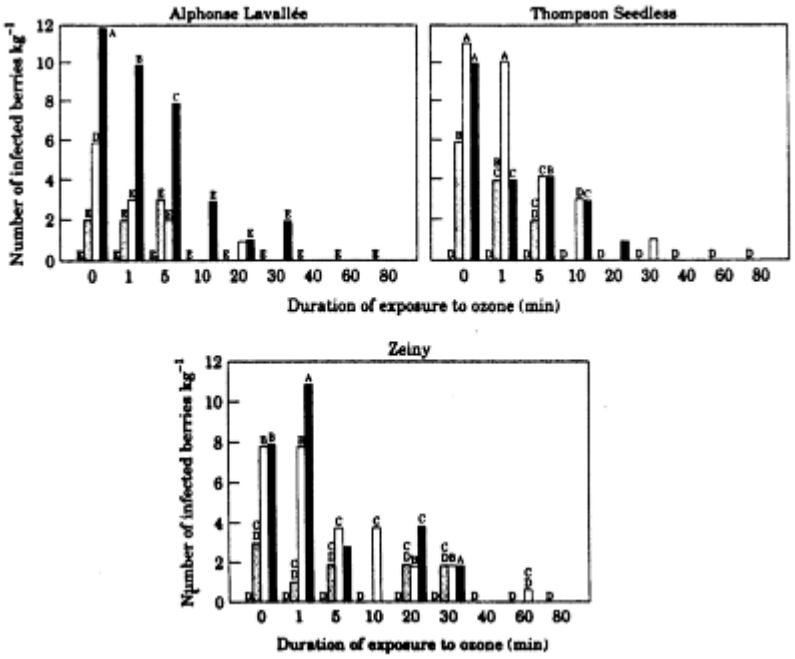


Figure 2.4.3. Decay severity caused by fungi on berries of cvs. Alphonse Lavellée, Thompson Seedless and Zeiny, treated with 70% ethanol prior to ozone exposure (~ ∴); exposed only to ozone (~); inoculated with *Rhizopus stolonifer* before (~) and after ( ) exposure to ozone for different periods of time (decay assessment made after 6 days at 20EC). Columns with different letters in each figure indicate significant differences by Duncan's multiple range test (P # 0.05) (Sarig et al., 1996).

A Comparison of SO_2 and Ozone for the Control of Berry Decay During and Following Cold Storage: Experiments were performed to compare a pre-storage exposure to ozone with a continuous SO_2 supply during storage from a two stage generator. Decay severity on untreated fruit stored for 5 weeks at 0EC and 3 days at 20EC was high, but did not increase with longer storage periods. It was caused mainly by two fungi -- *B. cinerea* and *R. stolonifer*, the former during storage and the latter during shelf life. Results showed that both ozone and SO_2 treatments decreased decay severity significantly. Although the differences between the two treatments were not significant, the extent of decay in the SO_2 treatment always was less than in the ozone-treated fruit and the overall appearance of SO_2- treated fruit generally was superior. After 9 weeks of storage, the decay severity and fruit quality were equivalent in both lots of treated fruit, which were significantly superior to untreated controls.

Discussion

The effectiveness of a single, short post-harvest exposure of table grapes to ozone for the control of storage cluster decay apparently is due to both its direct fungicidal effect on the decay-causing organisms and its ability to elicit stilbene phytoalexins in the grape berries. A 10-min exposure to ozone was at least as effective as immersing the fruit in ethanol for reducing the microbial population inhabiting harvested berries. The bactericidal effect is probably irrelevant, as no

known decay is caused by bacteria. With regard to yeasts, they are often found to inhabit decayed fruit, but have not been shown to be the initial causal organisms of decay. Their reduction on the fruit surface may even be detrimental, since many of the yeast species found on plant surfaces are beneficial for biological control of decay-causing fungi.

Total sterilization of the fruit surface after harvest presumably can provide complete protection of the fruit from decay development during prolonged storage, as evidenced by the effect of immersion in 70% ethanol (Figures 2.4.2 and 2.4.3). Even when ethanol alone did not completely eliminate all microorganisms from the fruit surface, decay control was almost complete. This is somewhat surprising, as one would expect decay to develop in storage either front reinfection from the spore-laden atmosphere or from latent infections by *B. cinerea.*

A good correlation call be observed between the size of' the fungal population remaining on the fruit following ozone treatment and the extent of storage decay on non-inoculated fruit (Figures 2.4.2 and 2.4.3). However, the control of decay development in fruit inoculated with *R. stolonifer* after exposure to ozone (Figure 2.4.3), points to elicitation of a defense mechanism. In this instance, decay control can be attributed to ozone elicitation of resveratrol and pterostilbene, both of which are correlated with the relative resistance of grapes to fungal decay.

The results of a trial conducted with several boxes of fruit, cold-stored for up to 9 weeks, indicate that a short-term post-harvest exposure of grapes to ozone might be a commercially satisfactory alternative to SO_2. Not only was decay controlled by ozone as effectively as by SO_2, but the quality and freshness of the treated fruit were superior to those of the untreated control fruit. However, the SO_2- treated fruit generally was of better appearance due to the bleaching of the stems and pedicels, which imparts a fresh look even when these parts are actually relatively dry.

2.4.7 Lyons-Magnus (Sept. 28, 1999) – Strawberries and Frozen Strawberry Topping

The following material was submitted by Lyons-Magnus (Fresno, CA) to the EPRI Agricultural and Food Alliance in regarding antimicrobial data on fresh strawberries during treatment by ozone.

Ozonated water is sprayed on fresh strawberries as a sanitizing agent to reduce the overall microbial load before freezing of the berries. This process has been in use at this plant since June 1998. Ozonated water also is used for treating frozen strawberry toppings. Specific data are presented in the following two tables (Tables 2.4.5 and 2.4.6) It is clear that washing raw strawberries with water containing an average of 2.7 ppm of ozone reduces levels of *E. coli* and coliform organisms, standard plate counts and yeasts and molds by considerable amounts. For example, SPC organism levels are reduced on average from 17,767 in raw strawberries to 987. Likewise, yeast/mold counts are reduced from an average of 56,500 to 1,304 (Table 2.4.5). Similar results are obtained in Table 2.4.6).

Table 2.4.5. Raw Strawberries and Frozen Strawberry Topping Micro Data. Week of July 13-18, 1999 (Lyons-Magnus, 1999)

Date	Time	Raw			Finished			Tsunami	Ozone
		E. coli/-coli	SPC	Yeast/-Mold	E. coli/-coli	SPC	Yeast/-Mold		
	Specs	Neg/<10	<50000	<2000	Neg/<3	<10000	<2000	ppm	ppm
13-July	7:00 AM	Neg/<10	4000	TNTC	Neg<10	1000	800	21	2.52
	9:30 AM	Neg/3000	45000	TNTC	Neg<10	1300	900	21	2.52
	5:15 PM	Neg/TNTC	TNTC	TNTC	Neg<10	2000	1100	21	2.34
	7:30 PM	Neg/200	600	12000	Neg<10	1400	1300	21	2.6.2
	9:50 PM	Neg/15000	50000	160000	Neg<10	330	1700	21	2.47
	11:15 PM	Neg/<10	800	TNTC	Neg<10	300	1200	21	3.04
14 July	2:00 AM	Neg/200	TNTC	TNTC	Neg<10	60	700	21	2.88
	2.35 PM	Neg/12000	TNTC	TNTC	Neg<10	800	1900	12	2.36
	5:30 PM	Neg/120	1400	9000	Neg<10	1500	950	4	2.36
	7:45 PM	Neg/2100	TNTC	TNTC	Neg<10	600	750	4	2.30
	9:35 PM	Neg/1300	TNTC	TNTC	Neg<10	300	1400	3	2.38
15 July	1:20 PM	Neg/7000	TNTC	TNTC	Neg<10	60	600	9	2.86
	2:20 PM	Neg/900	80000	16000	Neg<10	210	500	9	2.86
	3:20 PM	Neg/700	TNTC	TNTC	Neg<10	4100	2000	8	2.86
	5:35 PM	Neg/800	TNTC	TNTC	Neg<10	140	1600	3	3.15
	7:45 PM	Neg/1200	TNTC	TNTC	Neg<10	520	1400	3	2.43
	9:45 PM	Neg/60	TNTC	TNTC	Neg<10	600	1600	5	2.71
16 July	6:45 AM	Neg/1200	TNTC	TNTC	Neg<10	200	900	18	2.75
	9:45 AM	Neg/400	11000	TNTC	Neg<10	150	1800	14	2.75
	3:05 PM	Neg/80	16000	TNTC	Neg<10	50	400	3	2.36
	5:45 PM	Neg/1800	7600	TNTC	Neg<10	1000	1700	4	2.54
	7:25 PM	Neg/300	2700	90000	Neg<10	500	2000	5	2.43
	9:25 PM	Neg/200	14000	70000	Neg<10	900	2100	5	2.71
17 July	6:50 AM	Neg/TNTC	TNTC	TNTC	Neg/3000	1200	1800	5	2.96
	4:00 PM	Neg/TNTC	TNTC	TNTC	Neg/900	2000	1300	4	2.85
	5:05 PM	Neg/TNTC	TNTC	TNTC	Neg/500	1800	1100	4	2.71
	7:15 PM	Neg/TNTC	TNTC	TNTC	Neg/400	1500	2500	4	2.73
	9:25 PM	Neg/10	30000	80000	Neg/3500	5400	2700	4	3.14
	11:10 PM	Neg/TNTC	TNTC	TNTC	Neg/1200	700	1200	5	2.78
18 July	6:55 AM	Neg/200	400	TNTC	Neg/<10	300	450	11	2.79
	8:25 AM	Neg/12000	TNTC	TNTC	Neg/200	700	400	11	2.79
	11:25 AM	Neg/200	3000	TNTC	Neg/<10	900	700	12	2.79
	3:20 PM	Neg/TNTC	TNTC	TNTC	Neg/<10	700	1400	4	2.80
	5:05 PM	Neg/60	TNTC	15000	Neg/<10	350	1500	4	2.91
	Average		17767	56500		978	1304	9.5	2.7
	Std. Dev.		23538	53676		1118	596	6.9	0.2
	Maximum		TNTC	TNTC		1000	1500	21.0	2.9
	Minimum		4000	15000		350	800	4.0	2.5

Table 2.4.6. Raw Strawberries and Frozen strawberry Topping Micro Data. Week of July 6-11, 1999 (Lyons-Magnus, 1999)

Date	Time	Raw			Finished			Tsunami	Ozone
		E. coli/-coli	SPC	Yeast/-Mold	E. coli/-coli	SPC	Yeast/-Mold		
	Specs	Neg/<10	<50000	<2000	Neg/<3	<10000	<2000	ppm	ppm
6-July	6:45 AM	Neg/TNTC	40000	TNTC	Neg<10	600	1100	4	2.56
	9:15 AM	Neg/25000	60000	TNTC	Neg<10	10	<10	5	2.56
	1:15 PM	Neg/8000	6000	1800	Neg<10	30	20	6	2.62
	5:30 PM	Neg/2500	8000	900	Neg<10	100	20	8	2.59
7 July	1:05 PM	Neg/5000	800	TNTC	Neg<3	3	<3	8	2.77
	2.35 PM	Neg/40	1200	24000	Neg<3	12	<3	8	2.77
	5:22 PM	Neg/4000	10000	35000	Neg<3	6	<3	5	2.93
	7:20 PM	Neg/20	30000	TNTC	Neg<10	800	1900	6	2.98
8 July	2:30 AM	Neg/<10	600	71000	Neg<10	650	1600	6	2.98
	6:50 AM	Neg/<10	5000	TNTC	Neg<10	2000	1400	6	2.79
	9:20 AM	Neg/<10	1200	20000	Neg<10	<10	<10	7	2.82
	2:50 PM	Neg/<10	6000	40000	Neg<10	<10	<10	7	2.72
	5:40 PM	Neg/400	TNTC	TNTC	Neg<10	180	500	5	2.70
9 July	1:20 AM	Neg/300	4000	12000	Neg<10	900	2500	5	2.88
	5:15 PM	Neg/150	TNTC	TNTC	Neg<10	800	700	5	2.51
	7:30 PM	Neg/TNTC	20000	TNTC	Neg<10	580	1100	5	2.55
	9:35 PM	Neg/TNTC	18000	TNTC	Neg<10	900	800	5	2.51
	11:00 PM	Neg/1500	600	90000	Neg<10	1900	1700	5	2.76
10 July	1:15 AM	Neg/TNTC	TNTC	TNTC	Neg/<10	2200	1500	5	2.76
	2:20 AM	Neg/<10	80000	TNTC	Neg/<10	1480	2000	5	2.81
	7:00 AM	Neg/TNTC	TNTC	TNTC	Neg/<10	1000	800	5	2.78
	8:30 AM	Neg/TNTC	TNTC	TNTC	Neg/<10	750	600	6	2.78
	2:30 PM	Neg/TNTC	TNTC	TNTC	Neg/<10	200	150	7	2.78
	7:40 PM	Neg/TNTC	TNTC	TNTC	Neg/<10	350	1020	5	3.09
11 July	1:15 PM	Neg/<10	TNTC	TNTC	Neg/<10	100	1700	6	3.01
	7:40 PM	Neg/<10	TNTC	TNTC	Neg/<10	300	900	5	2.68
	10:05 PM	Neg/<10	TNTC	TNTC	Neg/<10	200	500	4	2.36
	Average		17141	32744		642	1074	6	2.74
	Std. Dev.		23063	30496		654	678	1	0.17
	Maximum		TNTC	TNTC		2200	2500	8	3.09
	Minimum		600	900		3	20	4	2.36

2.4.8 U.S. Patent Application No. 09/217.581, Tahoe Food Technology, Inc., Sparks, NV (22 December 1998) – Navel Oranges

The inventive method comprises applying a continuous stream of "Ox" gas (which contains ozone) to a material in a sealed biological burden reduction chamber. The continuous stream of Ox gas is prepared in an Ox generation cell, which contains a means for generating the Ox gas at a pressure less than about 20 lb/in^2 using, for example, one or more of the following: corona discharge, high frequency electrical discharge, ultraviolet light, x-ray, radioactive isotope and electron beam.

The inventive apparatus comprises:

1. a biological burden reduction chamber,

2. a vacuum pump coupled to the biological burden reduction chamber,

3. an Ox generation cell, wherein the Ox generation cell contains a means for generating Ox at pressure less than about 20 lbs/in^2,

4. a first control valve coupled to the biological burden reduction chamber and the Ox generation cell, wherein the first control valve is capable of permitting Ox to be drawn from the Ox generation cell into the biological burden reduction chamber, and

5. a second control valve coupled to the biological burden reduction chamber.

The following data are given in this patent application for the Ox treatment of navel oranges for the control of bean thrips, red scale and fuller rose beetle:

(1) Parameters

O_3 Concentration (by weight)	:	4.0%
CO_2 Concentration (by weight)	:	10.0%
Argon Concentration (by weight)	:	1.0%
Chamber Temperature	:	20EC
Relative Humidity	:	30%
Time (VVP)	:	2 hours
Pressure	:	9.5 psia

(2) Results

All adults, larvae, and eggs (Fuller Rose Beetle only) were killed in the process. The treated fruit was incubated for 28 days after treatment to ensure that all three life cycles had been destroyed.

2.4.9 McLoughlin (2000) – Caramel Apples

The Tastee Apple Company has a caramel apple processing plant in Ohio that historically used apple wash water containing chlorine. However, with the assistance of grant funcing from its electric utility (American Electric Power) and the Electric Power Research Institute, Tastee has replaced its chlorinated water system with an ozone treatment system at this plant. This has resulted in lowered disposal costs and has saved more than 12,000 gallons of water per week.

This food processor starts with Jonathan or Empire apples, which arrive from the field in bins. The plant used a 2000 gallon recirculating chlorinated-water channel, or flume, to float 950 pounds of apples out of each bin and move them toward a conveyor for further processing. The water reduces bruising and washed the apples at the same time. The flume can handle about 40,000 apples per hour. After washing, the apples are coated with sweet brown or red caramel, then dipped in either peanuts or coconut.

The recirculating flume water had to be dumped daily because it accumulated high levels of soil and organics. And even though the flume water was chlorinated, trace bacteria, yeast and molds remained that shortened the apples' shelf life.

With the new ozonation system, water is circulated through the flume at about 600 gal/min. To ozonate the water, a side stream of about 60 gpm is first filtered through a filter with intermittent backwash capability. Following filtration, gaseous ozone is injected into this side stream (5 lbs/day ozone generator using dried air). After about 10 minutes exposure to the ozonated water, the apples are conveyed to the sorting, caramel and packaging areas.

For safety purposes, an ozone detection unit continuously monitors the amount of ambient ozone in the plant air in the area adjacent to the flume to ensure that the concentration of ozone in plant air in this area does not exceed state standards. The ozone concentration is maintained at about 0.05 to 0.15 ppm.

Since the installation of the ozonation system in 1999, Tastee Apple now can reuse the same water for seven days, saving more than 12,000 gallons per week. Since the ozonation system runs 24 h/day, it is able to reduce the amount of waste in the flume water – particularly during the night when the apples are not moving – to less than 50 mg/L. Less waste means less disposal costs, particularly the costs associated with high BOD and suspended solids.

According to Tastee Apple's plant management, ozone treatment has reduced the yeast and mold count in the water, resulting in cleaner apples and a longer shelf life for the caramel apples. As a result, fewer consumer complaints of moldy apples have been received since installation of the ozonation system.

2.5 VEGETABLES

2.5.1 Kondo et al. (1989) – Chinese Cabbage

Summary: As a possible means of bacterial control and antibacterial protection in the field of food hygiene, the effects of sterilization by ozone water and ozone ice treatment were examined using seven different strains of bacteria, including various causal agents of food-poisoning. The results obtained were as follows.

1. All test organisms except for spore-forming bacteria such as *Bacillus subtilis* were sterilized 100% within 10 min by ozone water with an ozone concentration of 2.3 mg/L.

2. The bactericidal activity of ozone (2.3 mg/L) prepared in phosphate buffer solution (pH 7.2) was slightly superior to that of ozone water.

3. The ozone ice (2.3 mg/L) reduced viable cell count of the test microorganisms to 10^2-10^3/mL, including spore-forming bacteria.

4. The effects of ozone water against contaminants on the surface of Chinese cabbage were tested bacteriologically. Chinese cabbages were placed in water containing 2.3 mg/L ozone at 6EC for 10, 30 and 60 min, respectively. The results indicated that, for long-term preservation, contaminants would have a tendency to be reduced. The total bacterial counts were reduced by over 90%.

5. The effects of sterilization using ozone water were superior against bacteria suspended in water or phosphate buffer. However, it was thought to be difficult to kill bacteria adhering to the surfaces of various foods. Accordingly, to enhance the sterilization effects of ozone water against surface contaminants, it seems necessary to manually abrade the bacteria from the surface into the solution.

Materials and Methods

Bacteria Tested: The following seven strains of bacteria were tested:

Escherichia coli NIHJ (*E. coli*), *Staphylococcus aureus* 209P (*S. aureus*), *Micrococcus luteus* ATCC-9341 (*M. luteus*) *Salmonella typhimurium* R-124 (*S. typhimurium*), *Salmonella pullorum* SANK 73987) (*S. pullorum*), *Bacillus subtilis* ATCC-11778 (*B. cereus*). The bacteria were cultured aerobically in Heart Infusion Broth (HIA; Difco) medium at 37EC for 24 hours; then the bacterial cultures were washed twice using sterilized distilled water or sterilized 1/15 M phosphate buffer (pH 7.0) in a refrigerated centrifuge (for 30 minutes each at 6,000 rpm), and the washed liquids were used for the test. The culture media used for determination of the bacterial counts were EMB medium (H water) for *E. coli* and Heart Infusion Agar (HIA; Difco) for the rest of the bacteria tested. The washed liquids were treated with ozone water or ozone ice for the varying test times and then 1:10 dilutions of the liquids which received the ozone treatment were inoculated by streaking on plates containing the agar medium and incubated at 37EC for 24 hours. At the end of the incubation, the plates were examined for the number of colonies, from which the viable bacterial counts/mL were calculated.

Ozone Generating System: The ozone generator (electrical discharge plate system) made by Kiyomoto Steel, Nobuoka City, Miyazaki Prefecture, was used. Whenever a higher concentration of ozone was required, oxygen was supplied from the oxygen bottle. The ozone generator was run until the ozone concentration reached the desired level and settled at the level. The ozone concentration levels were determined by measuring 2-3 times using a simple chemical color development method (color comparison method) (DPD Total Chlorine Reagent Powder Pillows for 5 mL Sample; HACH, P.O. Box 907, Ames, Iowa 50010, USA). The test was started after confirmation of the settlement of ozone concentration at the desired level.

Method of Measurement of the Bactericidal Effect of Ozone:

1) Bactericidal Effect of Ozone in Distilled Water: Each of the washed liquids containing the test bacteria was treated with a solution of 2.3 mg/L ozone in distilled water at 6EC for 0, 1, 5, 10 and 20 minutes and the viable bacterial count on each treatment was determined.

2) Bactericidal Effect of Ozone in Phosphate Buffer Solution: The bactericidal effect of ozone in phosphate buffer solution was determined using a solution of ozone in 1/15 M phosphate buffer (pH 7.2) in the manner identical with that of the ozone in distilled water solution.

3) Bactericidal Effect of Ozone Ice: The ozone ice was made by freezing to -80EC in a chamber of ultra-low temperature the solution of 2.3 mg/L ozone in distilled water at 6EC which is kept in a prechilled ice-making bath. The 2.3 mg/L ozone solution froze to ozone ice in a few minutes in the chamber of ultra-low temperature. The ozone concentration of the ozone ice was measured using the method of chemical color development prior to use in the test. One piece (10 mL) of ozone ice was transferred into a sterilized test tube to which the same volume of the washed liquid containing the test bacteria was added; then treated for 0, 1, 3, 5 and 10 minutes and the viable bacterial count on each treatment was determined.

4) Bactericidal Effect of Ozone Water on Chinese Cabbage: Chinese cabbage was purchased at the market and quartered into four equal portions and each portion of the cabbage was treated with the ozone water of 2.3 mg/L ozone concentration at 6EC for 0, 10, 30 and 60 minutes, respectively. A 50% slurry was prepared from each portion of the treated cabbage using a juicer-mixer and the slurry was used for determination of the viable bacterial count/g of the Chinese cabbage using the common bacterial inspection method (the following media were used to culture each strain of the microorganisms separated: Desoxy coleid medium for coli group, Manit NaCl medium for cocci, NGKG medium for cereus, KM with CW added agar medium for Welchii. and Saburo medium for true fungi).

Results

1. Bactericidal Effect of Ozone in Distilled Water: The liquids obtained by washing each of the 7 strains of bacterial culture were treated for varying test times with the ozone in distilled water at 2.3 mg/L ozone concentration and the viable bacterial count of each treatment was determined. The test results presented in Table 2.5.01 clearly show that the viable bacterial count was greatly decreased, on the order of 10^2 to 10^6 at the end of the 1 minute ozone treatment, while the prolonged ozone treatment for additional time beyond 1 minute reduced the

viable bacterial count only slightly. Sterilization by the ozone treatment of the spore-formers, *B. subtilis* and *B. cereus*, was not as effective as that of other test bacteria, and survivors of the spore-formers were found even after 10 minutes treatment with the ozone.

Table 2.5.01. Bactericidal effects on washed bacteria by distilled water with ozone (Kondo et al., 1989)

Organism	Treatment time (minutes)				
	0	**1**	**3**	**5**	**10**
E. coli	2.0×10^8	2.5×10^3	2.5×10^3	2.6×10^2	0
S. pullorum	1.5×10^8	1.0×10^2	0	0	0
S. typhimurium	2.0×10^8	8.0×10^2	4.0×10^2	4.0×10^2	0
M. luteus	1.0×10^7	5.0×10^5	5.0×10^3	0	0
S. aureus	1.8×10^8	2.8×10^2	0	0	0
B. subtilis	1.0×10^7	1.0×10^5	1.0×10^4	5.0×10^2	3.0×10^2
B. cereus	2.5×10^8	2.4×10^4	2.0×10^3	1.4×10^3	1.0×10^2

Number of viable microorganisms per mL.
0 = less than 1.0×10^2.
Ozone concentration in water = 2.3 ppm.
Water temperature = 6EC.

2. Bactericidal Effect of Ozone in Phosphate Buffer: The liquids obtained by washing each of the 7 strains of bacterial culture were treated for varying test times with the ozone in 1/15 M phosphate buffer solution (2.3 mg/L ozone concentration) and the viable bacterial count of each treatment was determined. The test results presented in Table 2.5.02 show that the bactericidal effect of ozone in phosphate buffer is nearly equal to that of the ozone in distilled water, although the ozone in phosphate buffer seems to be slightly more effective than the ozone in distilled water in reducing the number of viable bacteria. The spore formers, *B. subtilis* etc., were not completely killed even after 20 minutes treatment. The rate of viable bacterial count by the prolonged ozone treatment for additional time beyond 1 minute was very low.

Table 2.5.02. Bactericidal effects on washed bacteria by phosphate buffer with ozone (Kondo et al., 1989)

Organism	Treatment time (minutes)				
	0	**1**	**5**	**10**	**20**
E. coli	1.0×10^7	2.0×10^3	1.0×10^2	0	0
S. pullorum	1.0×10^8	1.0×10^2	0	0	0
S. typhimurium	2.0×10^7	1.0×10^2	1.0×10^2	0	0
M. luteus	4.8×10^6	6.0×10^3	0	0	0
S. aureus	2.0×10^6	2.0×10^2	0	0	0
B. subtilis	2.0×10^7	5.0×10^4	3.1×10^3	3.0×10^2	1.0×10^2
B. cereus	1.0×10^7	4.0×10^4	1.0×10^3	2.0×10^2	1.0×10^2
Number of viable microorganisms per mL. Ozone concentration in water = 2.3 ppm. Water temperature = 6EC.					

3. Bactericidal Effect of Ozone Ice: The treatment with the ozone ice (2.3 mg/L ozone concentration) of the test bacteria was not as effective as that of the ozone water in killing the test bacteria. As shown in the test results presented in Table 2.5.03, the viable bacterial count reduction after 20 minutes treatment was by the order of 10^2 to 10^3.

4. Sterilizing Effect of Ozone Water on Microorganisms Present in Chinese Cabbage: Chinese cabbage heads were quartered into four equal portions and immersed in ozone water (2.3 mg/L ozone concentration) for the varying test times. After the treatment, the viable microbial counts for total and also for each strain of the microorganisms present were determined and the results are presented in Table 2.5.04. The viable microbial count decrease after 60 minutes treatment was on the order of 10^1 for total and for *B. cereus* counts, on the order of 10^2 for *S. aureus* count. However, the ozone treatment was not very effective in sterilizing the anaerobes and true fungi. There was no recognizable discoloration of the Chinese cabbage before and after the ozone treatment.

DISCUSSION: Most of the bacteria tested were killed within one minute of the ozone water treatment. The ozone water treatment was very effective in killing the bacteria of gram-negative rods and gram-positive cocci, but sterilization by ozone treatment of the spore-formers tested, *B. subtilis* and *B. cereus*, was not as effective as that of the gram-negative rods and gram-positive cocci bacteria. The ozone in phosphate buffer was a little more effective than ozone in distilled water in killing the bacteria tested. Further tests with higher levels of ozone concentration and/or lengthened treatment time may be needed to develop more effective means of ozone sterilization.

Table 2.5.03. Bactericidal effects on washed bacteria by ozone ice (Kondo et al., 1989)

Organism	Treatment time (minutes)				
	0	**1**	**5**	**10**	**20**
E. coli	5.0×10^7	1.0×10^7	8.0×10^6	7.0×10^6	5.0×10^5
S. pullorum	1.0×10^8	2.5×10^7	1.8×10^6	8.0×10^6	1.0×10^5
S. typhimurium	1.8×10^8	1.5×10^7	1.3×10^6	1.3×10^6	1.0×10^5
M. luteus	3.0×10^7	1.0×10^7	8.0×10^6	4.0×10^6	2.0×10^5
S. aureus	3.0×10^8	3.0×10^7	2.6×10^6	1.0×10^6	3.0×10^5
B. subtilis	2.0×10^7	1.5×10^7	1.0×10^6	8.0×10^6	2.0×10^5
B. cereus	2.0×10^8	6.0×10^7	5.0×10^6	2.0×10^6	3.0×10^5
Number of viable microorganisms per mL. Ozone concentration in ice = 2.3 ppm.					

Table 2.5.04. Bactericidal effects of ozone water against contaminants on the surface of Chinese cabbage (Kondo et al., 1989)

Organism	Treatment time (minutes)			
	0	**10**	**30**	**60**
Total bacteria	2.5×10^5	1.2×10^5	9.8×10^4	5.5×10^4
Coli group	0	0	0	0
Staphylococcus sp.	2.0×10^5	1.1×10^5	1.0×10^4	2.5×10^3
B. cereus	6.0×10^3	2.0×10^3	1.4×10^2	1.0×10^2
C. perfringens	0	0	0	0
Anaerobes	2.1×10^2	1.7×10^2	1.1×10^2	1.0×10^2
Fungi	2.1×10^5	1.8×10^5	8.0×10^4	1.6×10^4
Number of viable microorganisms per mL. 0 = less than 1.0×10^2. Ozone concentration in water = 2.3 ppm. Water temperature = 6EC.				

The ozone ice treatment reduced the viable bacterial count on the order of 10^2 showing that ozone ice is much inferior to the ozone water in killing the bacteria tested. The ineffectiveness of the ozone ice in sterilization seems to be due to the 50% drop in ozone concentration of the ice with addition of the test liquid into the test tube containing the ozone ice and the additional drop resulting from the rapid decomposition into O_2 of the ozone gas coming out of the ice during the treatment.

Since no perfect sterilization method is available today, ozone sterilization may be considered as a method of sterilizing foods and removing any undesirable odor and color of the foods, without adding chemical preservatives, in order to supply safe foods to consumers. We believe that continuation of the study on ozone sterilization will be valuable.

2.5.2 Mitsuda et al. (1990) – Cucumbers

Abstract: The synergistic effect of ozone and carbon dioxide gases on the sterilization of food was investigated. Fresh cucumber and an agar plate of *Escherichia coli* were sterilized with a mixture of ozone and carbon dioxide gases in polyvinyl chloride film bags and stored. In both direct sterilization and the storage tests, the survival percentages for the mixed gases of ozone and carbon dioxide were lower than for those of the individual gases.

The reasons for this synergistic effect were considered to be that the bactericidal effect of ozone gas was retained during the storage period by the quenching effect of carbon dioxide gas to the chain reaction of ozone degradation, and by the bacteriostatic effect of carbon dioxide gas. The mixture of ozone and carbon dioxide gases sterilized both the surface and the inside of the food and the agar plate at the same time.

Materials and Methods: Fresh cucumbers were purchased from a local market, sliced to a thickness of 3 mm (about 50 g), and each slice packed in a bag made of polyvinyl chloride. The organisms (a representative strain which behaved biochemically as a typical member of *Escherichia coli*) were cultured in it medium comprising a standard agar for bacterial growth at 37EC for 48 hours.

Preparation of the Mixed Gas: The ozone gas was generated using commercial grade oxygen as the gas supply. The concentration of ozone gas generated in this apparatus was estimated to be 20-40 $g/m^{3.}$ Mixtures of ozone and carbon dioxide gases were prepared with a gas mixing tool by regulating the pressure of the gases to ratios of 3:1, 2:1, 1:1, 1:2 and 1 kg/cm^2 ozone (oxygen): 3 kg/cm^2 carbon dioxide.

Sterilization and Storage of Foods: In the food sterilization process, a bag of 20 cm x 1:1 cm, which had been made of a polyvinyl chloride film having no air-permeability, was used. After opening, each bag which contained the food was 80% sealed. The food in each bag had been exposed for 5 minutes to ozone gas, carbon dioxide gas or their mixture. Then the bag was completely sealed. Microbiological tests were carried out just after the 5-minute sterilization, and later after storage for 7 or 14 days in a refrigerator.

Microbiological Test for Sterilized Foods: Agar plate samples were prepared in accordance with a method of mixing and dilution. Ten grams of sliced cucumber meat were homogenized with 100 mL of physiological saline in an homogenizer. One mL of liquid was mixed with 10 mL of agar culture medium and then incubated at 37EC for 48 h. Each test was carried out in triplicate.

Sterilization of Bacterial Cells in the Agar Plate: To sterilize bacterial cells of *Escherichia coli*, plates containing bacterial cells were prepared by mixing one mL of properly diluted cells, which had been precultured in a brain heart infusion broth and 15 mL of agar culture medium in a glass Petri plate of 9 cm diameter. The sterilizing method was the same as in the case of the food described above. After opening, each 20 x l3 cm bag was 80% sealed. The plate in each bag had been exposed for 5 minutes to ozone gas, carbon dioxide gas or their mixture. Just after the 5-minute sterilization, plates were taken out of the bags and then incubated at 37EC for 48 h. For the storage tests, the bags were completely sealed after the 5-minute sterilization and the

plates were stored in the same gas at 37EC for 48 hrs. Calculations of the amount of bacteria in these plates were carried out after 48hrs.

Results and Discussion

Sterilization of Sliced Cucumber: A test was carried out to examine the sterilization of sliced cucumber with the gas mixture. As shown in Table 2.5.05, the number of bacteria in the samples was effectively reduced by sterilization for 5 minutes. This result differs from that of the beef samples (see Red Meats Section, same paper). It is assumed that this resulted because the cucumber slices had a larger surface area than a single slice of beef. For the cucumbers, the best results were obtained when the mixing ratios of ozone and carbon dioxide gas were 3:1 and 2:1.

After storage at 5EC for 14 days, the number of bacteria increased up to 200 times. In this case, the synergistic effect of ozone and carbon dioxide gases also was observed, especially for the mixing ratio of 2:1 and 1:1.

Sterilization of Bacterial Cells in an Agar Plate: To find the mechanism of this synergistic effect, a sterilization test for bacterial cells in an agar plate with ozone gas, carbon dioxide gas and a mixture of these gases was carried out. In the usual way, the number of the surviving bacterial cells was measured after ozone was bubbled through the bacterial suspension. In this study, however, the sterilization test was carried out on the bacterial cells located in the agar plate.

As shown in Table 2.2.04 (see Red Meats Section), the sterilization effect after 5 minutes was restricted, because the bacterial cells that were located on the surface of the agar plate were included as a part of the cells on the plate. Both in the five minutes of sterilization and the storage of the agar plate, the sterilization effect of some samples under the gas mixture was larger than that of samples under ozone and carbon dioxide gas in the single use. Desirable results were obtained when the mixing ratio of the ozone and carbon dioxide was 1:3 and 1:1.

In addition, the number of bacteria after exposure to carbon dioxide was greater than that for ozone, but the size of the colonies of bacteria was smaller for carbon dioxide. Furthermore, the colonies that resulted after exposure to ozone gas were found inside the samples.

Table 2.5.05. Survival of bacteria in different gases and gas mixtures just after exposure of sliced cucumber at room temperatures for 5 min at 5EC for 14 days (Mitsuda et al., 1990)

Kind of gas and mixing ratio	**Number of bacteria (survival %)**			
	just after exposure		**after storage**	
Control	30 x 10^4	100%	234 x 10^4	100%
Ozone gas	5.4	18	173	74
CO_2 gas	8.0	27	68	29
Mixed gas				
(O_3:CO_2, v/v)	0.9	3	140	60
3:1	0.8	3	17	7
2:1	1.3	4	17	7
1:1	1.1	4	210	90
1:2	9.6	32	119	51
1:3				

Table 2.2.04. Survival of *Escherichia coli* in different gases and gas mixtures just after exposure at room temperature for 5 min and at 37EC for 48 hours (Mitsuda et al., 1990)

Kind of gas and mixing ratio	**Number of bacteria (survival %)**			
	Just after exposure		**after storage**	
Control	440	100%	422	100%
Ozone gas	32	7	86	20
CO_2 gas	163	37	311	74
Mixed gas				
(O_3:CO_2, v/v)	56	13	125	30
3:1	55	13	122	29
2:1	300	68	0	0
1:1	310	70	124	29
1:2	5	1	166	39
1:3				

After the investigation on the mechanism of the interaction between carbon dioxide gas and protein, carbon dioxide binding sites in protein in the gas-solid phase system were revealed to be ,-amino, ∀-amino and guanidium groups. On the findings of these characteristics of carbon dioxide, the reasons for this synergistic effect observed in this study were considered as follows:

1. Ozone was reported to be bactericidal rather than bacteriostatic and carbon dioxide gas was known to be bacteriostatic. The synergistic effect could not be assumed to be caused by the simple summation of the bactericidal effect of ozone gas, and the bacteriostatic effect of carbon dioxide gas, because the optimal conditions for the synergistic effect were obtained in a restricted ratio of both gases.

2. The bactericidal effect of ozone gas was assumed to be retained during these storage periods by the quenching effect of carbon dioxide gas to the chain reaction of ozone degradation. Decrease of the bacterial number for the storage period was assumed to be caused by the high solubility and the bacteriostatic effect of carbon dioxide gas to the bacterial cells which were located not only on the surface but also inside the agar plate.

2.5.3 Kim et al. (1993) – Chinese Cabbage Kimchi (in Korean with English abstract and English figures)

Abstract: This work was conducted to study the use of *L. acidophilus*, which exists in human intestine for the fermentation of Chinese cabbage kimchi. The changes in vitamins, the number of microflora and sensory quality were observed during fermentation after the microflora which was not related to kimchi fermentation was eliminated by treatment with ozone water or ozone gas. The growth rate of *L. acidophilus* in the cabbage juice was higher than that in MRS broth. The growth of *L. acidophilus* was slightly promoted by adding 1-2% hot pepper powder while that was inhibited by ginger and garlic. Therefore, it was shown that the regulation of fermentation was possible by addition of spices. The result of treating spice with ozone gas and ozone water 6 mg/L/sec for 1 hour was that the survival ratio of total microflora was 6-20%. When *L. acidophilus* was added to materials after ozone treatment, the fermentation rate was improved and the polysaccharides in the cell wall were used when the usable free sugar was all consumed. The contents of vitamin B1 and C in the ozone-treated kimchi was higher than in the control.

The following Tables and Figures are in English in this article:

Table 2.5.06 shows the total microbial counts remaining after ozone treatment of the various ingredients used to prepare kimchi.

Figure 2.5.01 shows the growth rate of *L. acidophilus* in Chinese cabbage juice during fermentation. Figure 2.5.02 shows the changes in numbers of aerobic bacteria in the kimchi prepared with ozone-treated cabbage and inoculation of *L. acidophilus* during fermentation at 25EC. Figure 2.5.03 shows the changes in numbers of lactic acid bacteria in the kimchi prepared with ozone-treated cabbage and inoculation of *L. acidophilus* during fermentation at 25EC.

Table 2.5.06. Total Microbial Counts Remaining after Ozone Treatment of the Various Ingredients Used to Prepare Kimchi (Kim et al., 1993)

Ingredient	Non-treatment (CFU x 10^4/g-f.w.)	Treatment (CFU x 10^4/g-f.w.)
Chinese cabbage [1]	199.56 (100) [3]	35.92 (18)
Hot pepper powder [2]	68.95 (100)	13.79 (20)
Garlic [2]	92.58 (100)	19.44 (21)
Ginger [2]	90.32 (100)	5.42 (6)
Green onion [1]	65.45 (100)	8.51 (13)
Leek [1]	84.07 (100)	12.61 (15)

[1] The materials were cleaned by ozone water.
[2] The materials were cleaned by gaseous ozone.
[3] Parenthesis denotes percent of non-treatment.

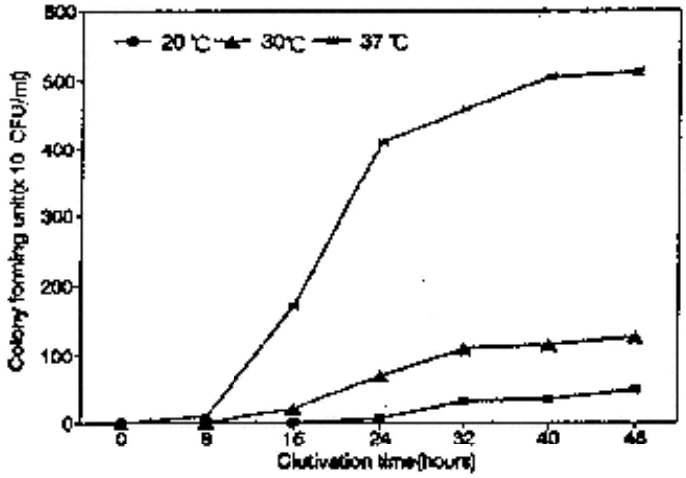


Figure 2.5.01. Growth rate of *L. acidophilus* in Chinese cabbage juice during fermentation (Kim et al., 1993).

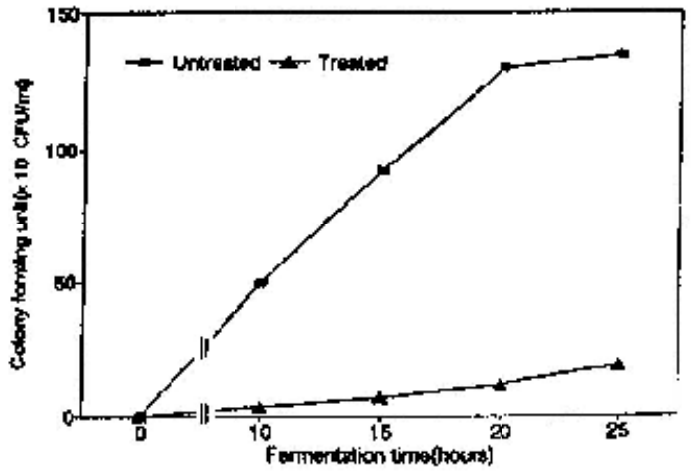


Figure 2.5.02. Changes in numbers of aerobic bacteria in the kimchi prepared with ozone-treated cabbage and inoculation of *L. acidophilus* during fermentation at 25EC (Kim et al., 1993).

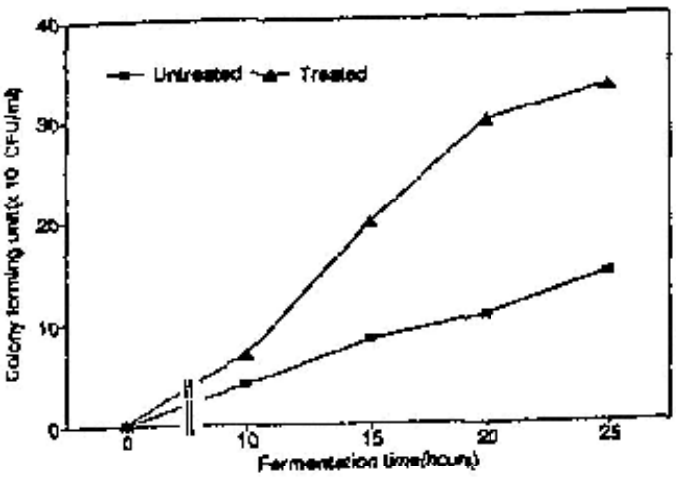


Figure 2.5.03. Changes in numbers of lactic acid bacteria in the kimchi prepared with ozone-treated cabbage and inoculation of *L. acidophilus* during fermentation at 25EC (Kim et al., 1993).

2.5.4 Hampson et al. (1994) – Broccoli, Broccoflower and Carrots

Abstract: Using existing and newly acquired equipment, a system was constructed for the testing of ozone (O_3) for use as a sanitizing agent in food processing. The intended use of the system is to demonstrate that ozone is an effective and efficient germicide for the treatment of raw agricultural commodities. The system has the capacity to ozonate 200 gallons of water which then can be used to wash produce, reducing the microbial load through direct contact

between the ozone in the water and the microbes on the produce. The system is designed to model a flume wash system with a cascading water supply. A one horsepower motor powers the water flow and ozone levels are monitored continuously via in-line ORP probes. Ozone levels are confirmed using the HACH colorimetric assay. The corona discharge ozone generator is combined with an oxygen generator yielding ca. 15 grams O_3 per hour. This allows for sufficient variation in test parameters, such as organic loading, incoming microbial load, the decay rate of O_3, specific surface area of the food, water flow, and temperature. All experimental samples are tested immediately on-site for Aerobic Plate Count, Yeast and Mold, Coliforms, or Mesophilic Spores. Initial studies indicate that a three log-fold reduction in microbial load is possible with a ten minute contact time (CT: where CT = mg/L O_3 x minutes). In order for the process to be commercially feasible, the CT must be reduced by at least 50% without sacrificing the germicidal efficacy. From an engineering standpoint, the goal is to develop specific CT values for different commodities based upon the above-mentioned variables. These studies are of significance to the government for the approval of O_3 as a food-contact sanitizing agent; to the industry as a chlorine alternative; and to the consumer, and everyone, from a food safety perspective.

Sample Treatment: Approximately 1000 grams of each sample (broccoli, broccoflower and carrots) was weighed and placed into presanitized nylon mesh bags. These samples were treated in ozonated water for one, five or ten minutes. The control sample was washed for a period of five minutes in non-ozonated water. After treatment, samples were cut into small segments using a sanitized knife. A random 50 gram sample was blended with 450 mL of peptone broth (1:10 dilution) and scheduled microbial tests were performed within 30 minutes of washing.

Microbial Tests: Aerobic Plate Count (APC) and yeast and mold (using DRBC agar; Oxoid) tests were performed using standard microbiological methods (FDA BAM, APHA).

Results: The following figures depict the results of using ozone in the range of 0.64 to 1.11 ppm on broccoli, carrots and broccoflower: Figure 2.5.04 -- Reduction of microorganisms on broccoli using ozone at a concentration of 1.11 ppm; Figure 2.5.05 -- Reduction of microorganisms on carrots using ozone at a concentration of 0.64 ppm; Figure 2.5.06 -- Reduction of microorganisms on broccoflower using ozone at a concentration of 1.08 ppm. Under typical experimental conditions, anywhere from 1 to 3 microbial load reductions were seen. Broccoli shows the best reduction.

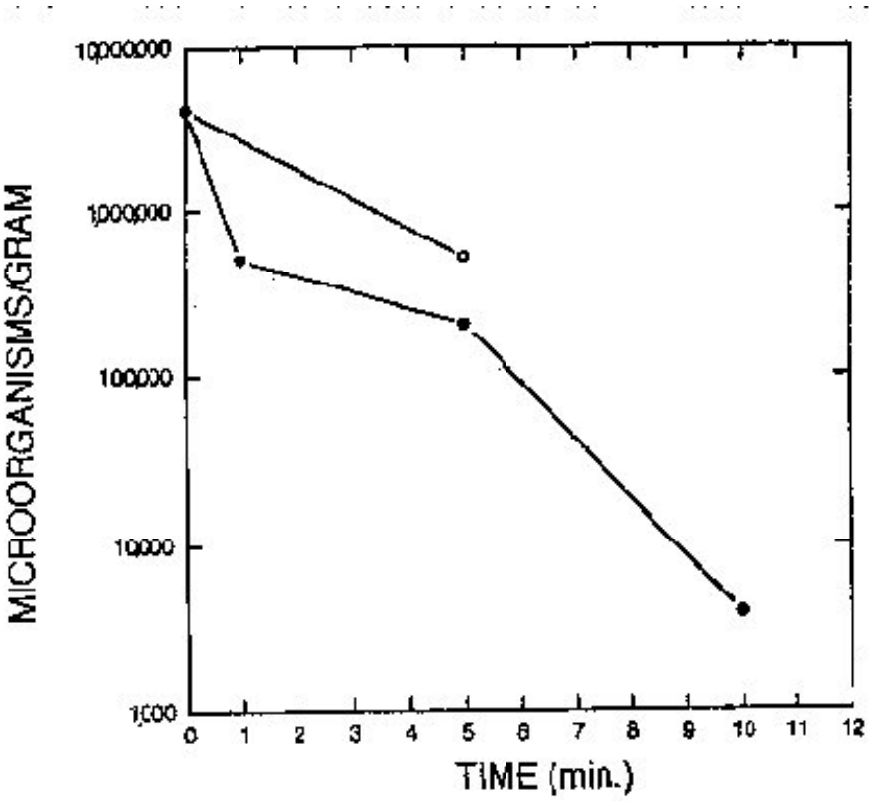


Figure 2.5.04. Reduction of microorganisms on broccoli using ozone at a concentration of 1.11 ppm (Hampson et al., 1994).

From the experimental data obtained, CT value equations were derived for broccoli, carrots and broccoflower. The following three figures show this information: Figure 2.5.07 -- Plot of CT values vs. log reduction for broccoli; Figure 2.5.08 -- Plot of CT values vs. log reduction for carrots; Figure 2.5.09 -- Plot of CT values vs. log reduction for broccoflower. The broccoli gave the smallest CT value per given log reduction. Each graph shows a high degree of correlation among the plotted CT points.

Using the same experimental protocol, chlorine was substituted for ozone as the germicidal agent. Comparing chlorination (Figure 2.5.10) to the results obtained using ozone, an additional 2-log reduction was realized for APC when ozone was used as the germicidal agent.

Conclusions: The results from this study support the following conclusions:

- Ozonation of raw agricultural commodities is a first order disinfection process.
- The results of this study give positive indications that ozone is an effective disinfectant.

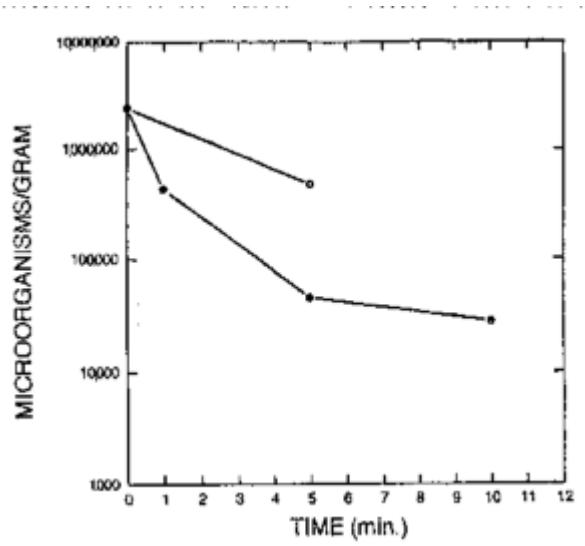


Figure 2.5.05. Reduction of microorganisms on carrots using ozone at a concentration of 0.64 ppm (Hampson et al., 1994).

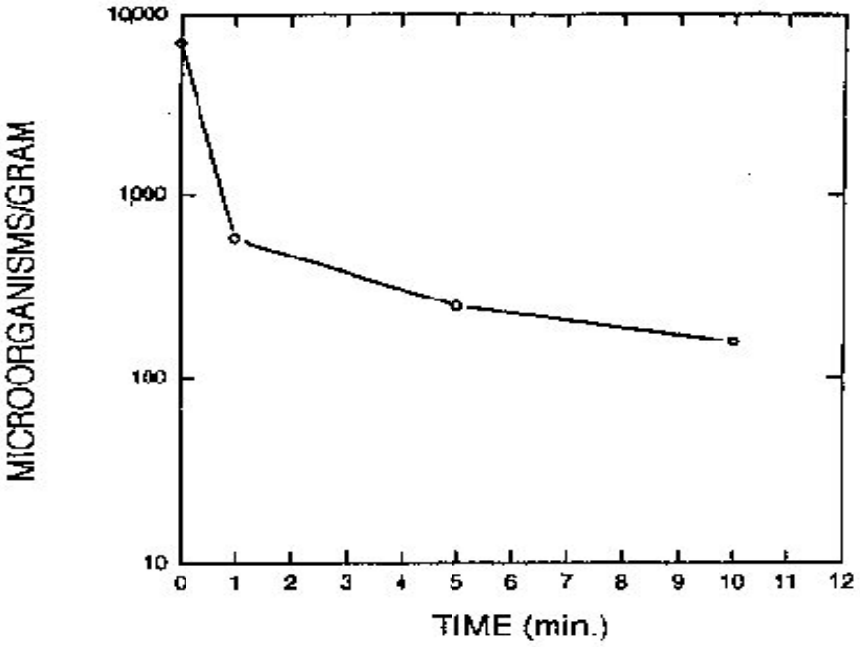


Figure 2.5.06. Reduction of microorganisms on broccoflower using ozone at a concentration of 1.08 ppm. (Hampson et al., 1994).

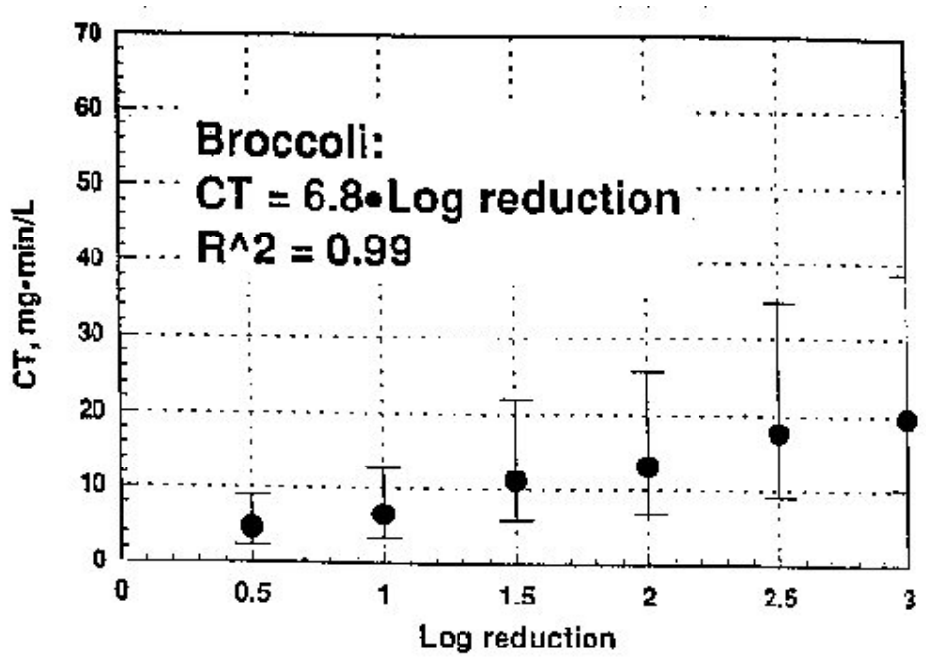


Figure 2.5.07. Plot of CT values vs. log reduction for broccoli (Hampson et al., 1994).

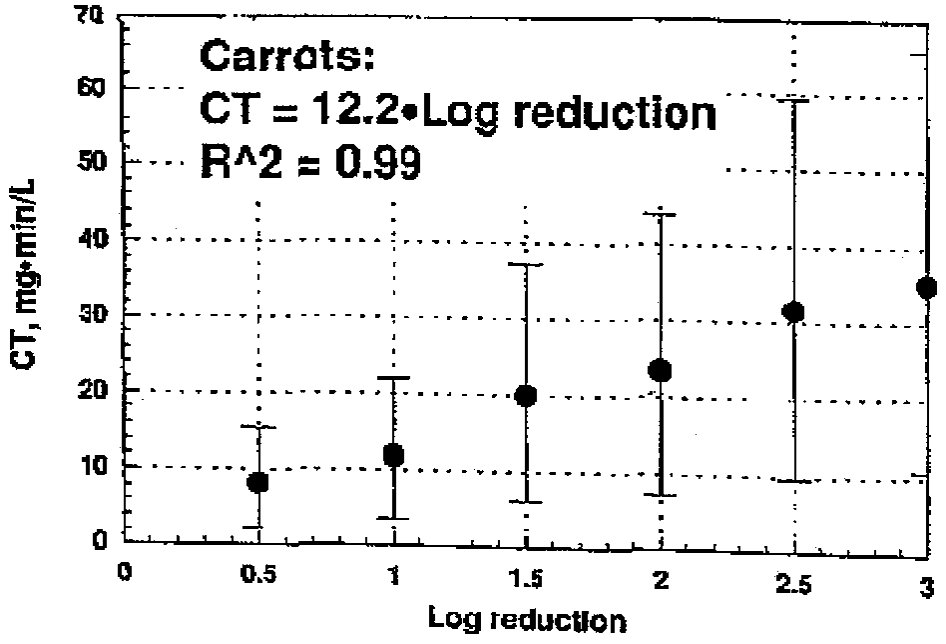


Figure 2.5.08. Plot of CT values vs. log reduction for carrots (Hampson et al., 1994).

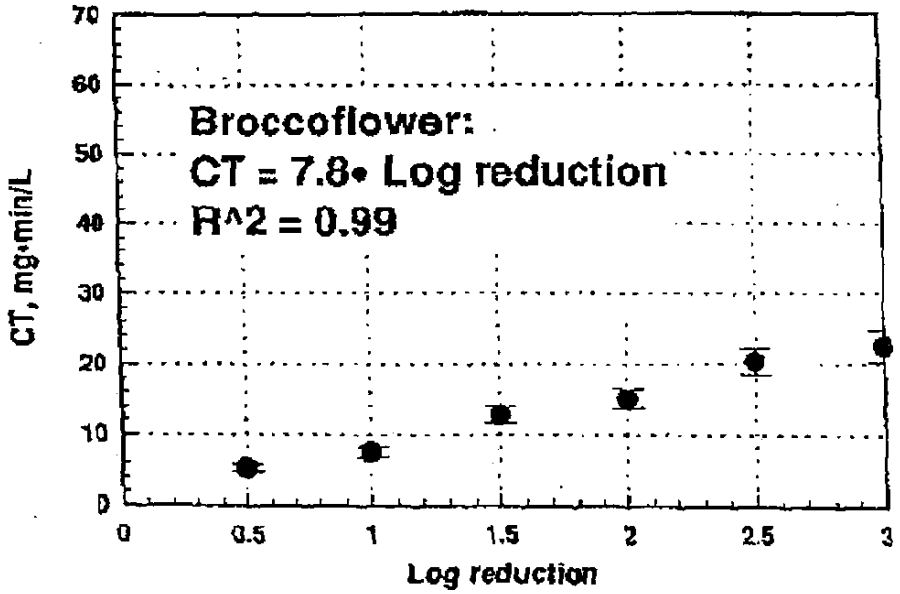


Figure 2.5.09. Plot of CT values vs. log reduction for broccoflower (Hampson et al., 1994).

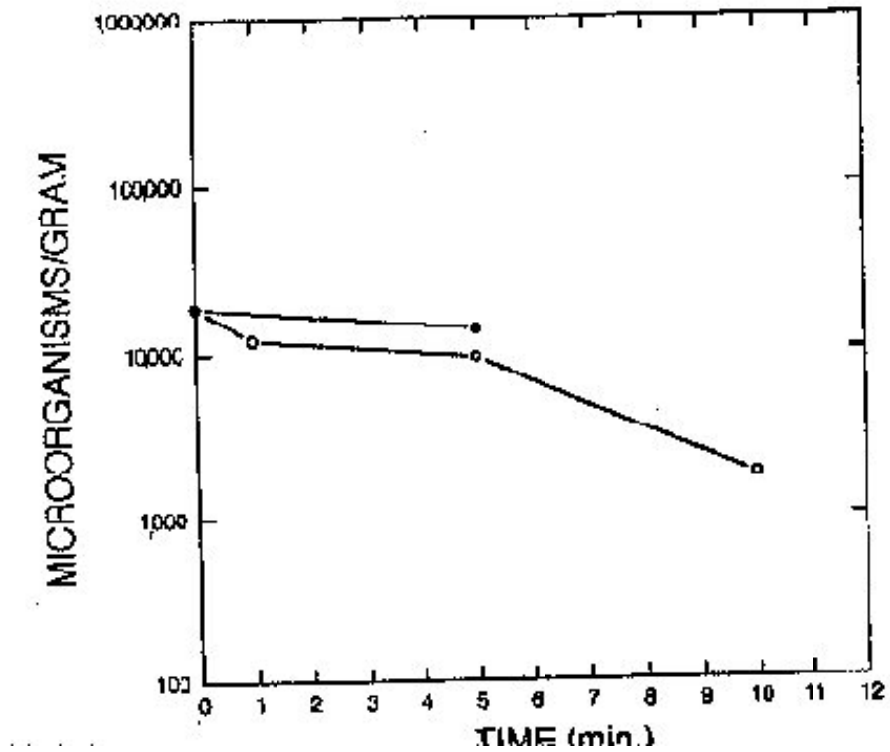


Figure 2.5.10. Chlorination of broccoli using 100 ppm of chlorine (Hampson et al., 1994).

- A number of independent factors influence the overall effectiveness of ozone as a disinfectant, e.g., water temperature, pH, ozone concentration, non-target demand substances, and specific surface area.

- Broccoli had the lowest CT values to achieve a given log reduction followed by broccoflower, then carrots.

- Preliminary results indicate that ozone proves to be a better disinfectant than chlorine under the conditions noted above.

2.5.5 Liew and Prange (1994) – Carrots

Abstract: Effects of ozone and storage temperature on carrots and two post-harvest pathogens, *Botrytis cinerea* Pers. and *Sclerotinia sclerotiorum* de Bary -- were investigated. Pathogen-inoculated and uninoculated whole carrots were exposed to an ozone concentration of 0 (control), 7.5, 15, 30, or 60 ΦL-liter^{-1}. Treatment chambers were flushed with a total flow rate of 0.5 L-min^{-1} (air and ozone) for 8 h daily for 28 days. The experiment was repeated twice at storage temperatures of 2, 8, and 16EC. The residual ozone concentration (ozone supplied--exhausted and reacted ozone) increased with ozone supply concentration but was less at higher storage temperatures. A 50% reduction of daily growth rates of both fungi at the highest ozone concentration indicated that ozone was fungistatic. Carrot respiration rate, electrolyte leakage, and total color differences increased with ozone concentration. Ozone-treated carrots were lighter (higher L* values) and less intense (lower chroma values) in color than control carrots.

The objectives of this study were 1) to determine the residual concentration of ozone, 2) to determine the effect of ozone on the two major storage pathogens of carrots, and 3) to observe ozone-induced changes in carrot physiology and quality during storage.

Methods and Materials:

'Vitabrite' carrots, obtained from a local grower (Berwick, N.S.), were hand-washed and stored at 0EC until use. Crown diameters of the carrots were from 3 to 4 cm.. An ozone generator (Tri-Ox Swindon, England) was set to produce 76.5 ΦL-liter^{-1} of ozone in air. Air containing ozone at flow rates of 0 (control), 0.05, 0.1, 0.2, or 0.4 liter-min^{-1} were blended with compressed air to produce ozone concentrations of 0, 7.5, 15, 30, or 60 ΦL-liter^{-1}, in a total flow of 0.5 liter-min^{-1} for each treatment. Ozone and compressed air flows were controlled with needle valves. Treatment chambers consisted of airtight 64-liter polyvinyl chloride containers placed in storage rooms set at 2, 8, or 16EC. The chambers were flushed continuously for 8 h daily for 28 days.

Ozone concentrations in the chambers were monitored during the treatment period with an ultraviolet-based detector with a measurement range of 0 to 100 ΦL liter^{-1} at 253.7 nm.

Disease: Isolates of *S. sclerotiorum* and *B. cinerea* were obtained from infected carrots in local storage. Fungal stock cultures and inoculum were maintained on potato dextrose agar (PDA). A 1.0-cm-diameter mycelial plug, obtained near the margin of a 4- to 5-day-old fungal culture, was placed in a wound of each carrot 1.0 ∀ 0.5 cm from the crown. The wound was a 1.0-cm diameter x 0.5-cm-deep depression created with a 1.0-cm diameter cork borer. Fungal growth

along the length of the carrot was measured once every 7 days for 28 days. A sample of 10 carrots was inoculated separately with each fungus for the 15 combinations of ozone concentration and temperature. The entire experiment was repeated twice.

A logarithmic (log_{10}) transformation was used to stabilize variance of fungal growth rates. Mean log values were back-transformed and are presented in parentheses in Table 2.5.07.

Results:

Disease: The fungal inoculum was applied to wounded carrots to ensure disease development. Fungal surface growth included spores, sclerotia and mycelia of *B. cinerea*, and only sclerotia, and mycelia of *S. sclerotiorum*.. Both fungi developed in the ozonated environment but exhibited varied mycelial growth rates and surface structure (sclerotia and spores) development on the carrots. At 2EC there were fewer surface structures present with increasing ozone concentration (data not shown). At the highest ozone concentration (60 ΦL liter^{-1}), water-soaked lesions (cellular necrosis) were the only indicators of fungal growth; however, mycelia developed on the carrots after removal from the ozonated atmospheres.

Growth rates of *B. cinerea* and *S. sclerotiorum* increased with temperature, with most of the increase occurring between 2 and 8EC (Table 2.5.07). A trend of decreased growth rate with increased ozone concentration was observed within each storage temperature. The growth rate for *B. cinerea* at 2, 8, and 16EC at 60 uL liter^{-1} ozone was 43% to 58% of the respective control treatment.

Although growth rate of *S. sclerotiorum* increased with storage temperature, the magnitude of the increase was reduced with higher ozone concentration (Table 2.5.07). The growth rate of *S. sclerotiorum* at 2, 8, and 16EC was reduced to between 44% and 63% at 60 ΦL liter^{-1} compared with the respective control treatments. The lower linear and quadratic regression coefficients at 2EC for *S. sclerotiorum* than at 8 and 16EC suggests that the higher residual ozone concentration at 2EC could have caused a greater reduction in fungal growth rate than those observed at 8 and 16EC. This effect was not observed for *B. cinerea*.

Discussion: Our results suggest that the efficacy of ozone as a disinfectant must be individually assessed for each commodity at its ideal storage temperature. Furthermore, the quantity of produce in storage and the system available for dispensing and measuring ozone must be considered.

The effect of ozone on *B. cinerea* and *S. sclerotiorum* was fungistatic and not fungicidal. Although some inhibitory effects were observed with ozone residual concentrations of 10 to 22 ΦL liter^{-1} at 2EC, these concentrations seemed to cause physical and physiological damage to carrots. Symptoms of physiological disruptions included increased respiration rates, electrolyte leakage, and color changes. Higher respiration rates with increasing ozone concentrations are likely an expression of abnormal metabolism or injury caused by ozone to the carrots. Further, alterations in appearance of ozone-treated carrots due to color changes and surface pitting may affect consumer appeal.

Table 2.5.07. Daily growth rate of *Botrytis cinerea* and *Sclerotinia sclerotiorum* on carrots at different temperatures and ozone concentrations in storage (Liew and Prange, 1994)

Storage temp (EC)	Ozone concentration (ΦL liter^{-1})					Regression coef.. (x 10^{-3})	
	0	7.5	15	30	60	Linear	Quadratic ^{L}
Botrytis cinerea ($\log_{10}$ mm day^{-1})							
2	-0.302 (0.499) [y]	-0.396 (0.401)	-0.459 (0.347)	-0.629 (0.237)	-0.667 (0.215)	-6.0	0.14
8	0.306 (2.02)	0.250 (1.78)	0.130 (1.35)	0.090 (1.23)	0.071 (1.18)	-6.3	0.12
16	0.379 (2.39)	0.323 (2.10)	0.117 (1.31)	0.044 (1.11)	0.036 (1.09)	-6.8	0.17
SEM [x] (n = 20, df = 12): 0.0417					SE (n = 2,		
Significant effects (P < 0.05): T, $O_{1,q}$ [w]					df = 3):	0.620	0.037
Sclerotinia sclerotiorum ($\log_{10}$ mm day^{-1})							
2	-0.275 (0.531)	0.331 (0.466)	-0.395 (0.403)	-0.498 (0.318)	-0.631 (0.234)	-5.9	0.05
8	0.472 (2.96)	0.389 (2.49)	0.315 (2.07)	0.345 (2.21)	0.271 (1.87)	-2.7	0.06
16	0.607 (4.05)	0.514 (3.27)	0.487 (3.06)	0.406 (2.55)	0.382 (2.41)	-3.4	0.10
SEM (n = 20, df = 12): 0.0497					SE (n = 2,		
Significant effects (P < 0.05): T, $O_{1,q}$, T x O_1					df = 3):	0.65	0.038

[L] Orthogonalized coefficient.

[y] Back-transformed mean (mm-day^{-1}).

[x] To compare effect of temperature within ozone supply concentration.

[w] T = temperature, O_1 = linear effect for ozone, O_q = quadratic effect for ozone.

If ozone is to be used for carrot storage, a balance must be found between preserving carrot quality and effective disease control. This study has demonstrated that reducing temperature from 16 to 2EC significantly reduced fungal growth of nonozonated carrots. Immediate ozonation may be unnecessary if carrots are properly harvested, washed, hydro-cooled, and cold-stored at the appropriate temperature. Although commercial carrot storages have air temperatures between 0 and 1EC, carrots stored in large bins may build up pockets of heat in the middle of the bins during long storage periods. These heat pockets are prime areas for fungal growth and development. It is often the combination of reduced resistance to infection by the carrots and poor heat removal that encourages pathogen proliferation. With proper air circulation and temperature control, ozonation may be introduced in the latter part of the storage period, when carrots are less resistant to fungal attacks. Even at low temperatures, diseases can spread by mycelial growth and spore production. Since carrot diseases often spread from one root to the next from a focus of infection or from contaminated storage bins, reducing surface fungal growth with ozone may limit the spread of pathogens. ***Based on our results, an ozone supply of 15 ΦL liter^{-1} for 8 h per day at 2EC could provide some disease protection with a minimum of physical and physiological damage***.

2.5.6 Zhuang et al. (1996) – Broccoli Florets

Abstract: Microbial spoilage is a major commercial concern with refrigerated fresh-cut vegetable and fruit products. Ozone is an effective agent for controlling microbial and fungal pathogens, and there are very few reports of ozone application for preservation of fresh-cut produce. The authors' objective was to evaluate impacts of short-term ozonated-water treatment on qualities of fresh-cut plant produce during refrigeration storage using fresh-cut broccoli. Broccoli florets were treated with ozonated-water (1 ppm) in a sealed container for 10 or 50 min, respectively, packaged in polymeric film, and stored under retail conditions (5EC) for 192 hr. Ozone effects on quality were evaluated by changes in surface color, nutrient (ascorbic acid and total carotenoids) levels and microbial growth. Ozone influence on broccoli physiology was determined by respiration rate, ethylene formation, protein contents and lipid peroxidation. Ozone treatment inhibited microbial growth without impact on quality over 4 days storage. However, after storage for 1 week, ozone resulted in losses in surface greenness and vitamin C with no effect on microorganisms. 50-min ozone treatment could better control microorganisms compared to 10-min treatment without further influence on other quality parameters of broccoli. No significant difference was found in carotenoids, respiration, protein and TBA values between the treated and control broccoli samples over storage, although reduced formation of ethylene was observed in ozone-treated broccoli florets following 22 hr storage.

Materials and Methods

Broccoli: Broccoli (cv. 'Iron Duke') florets were treated with 1 ppm ozonated-water for 10 or 50 min in a sealed container at 4EC. The florets then were packaged in microperforated polymeric bags and stored at 5EC for 192 hr. Samples of the broccoli florets were taken at 22, 96 and 192 hr, respectively.

Microbial Analysis: Total plate counts were made to estimate microbial growth in broccoli florets during low temperature storage. Duplicate samples (7 g) were ground with 30 mL sterile potassium phosphate buffer (0.1M) using a stomacher. The homogenates of each sample were deposited on plates to enumerate total microbial populations. Plates then were incubated at 37EC for 48 h prior to counting colonies.

Results

Microbial Growth: Over refrigerated storage, total plate counts increased by 10-fold in broccoli florets regardless of treatments (Figure 2.5.11). Ozone treatments inhibited microbial growth in broccoli florets in the first 96 hr storage. At 22 h interval, broccoli florets treated with 1 ppm ozonated-water for 50 min contained much less microorganisms than the other treated broccoli and the initial levels. Treatment of florets with tap water for 50 min increased microbial growth, however, no significant changes and difference was found in 10-min-treated samples. By 96 h, both ozone-treated florets had lower levels of microbial growth compared to the control groups. However, at the end of storage, no difference in microbial counts was determined among the treatments.

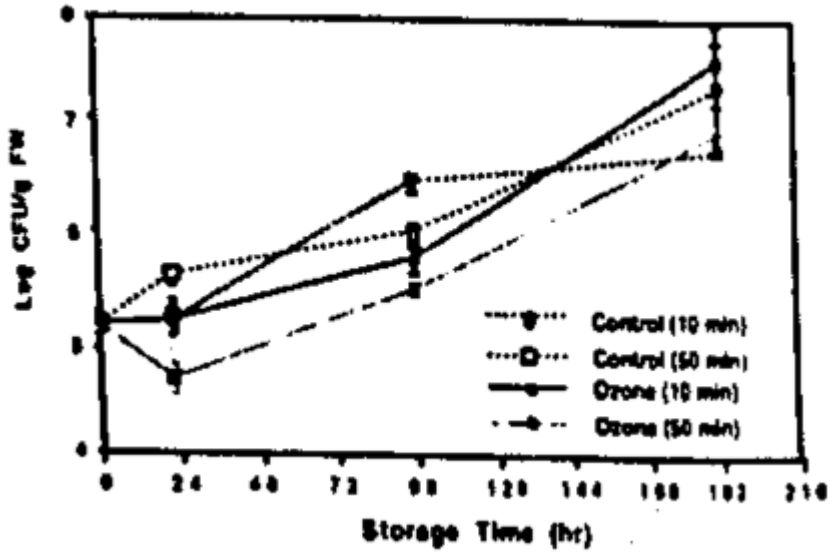


Figure 2.5.11. Effects of ozonated-water treatment on microbial growth levels of packaged broccoli florets during low temperature storage (Zhuang et al., 1996).

2.5.7 Hampson and Fiori (1997) – Broccoli, Broccoflower, Tomatoes

Abstract: The focus of this work is to determine the efficacy of ozone as a chlorine replacement in the sanitation of whole, fresh fruits and vegetables. A 200 gallon flume wash test system was constructed in the fruit and vegetables pilot plant of the Food Science and Nutrition Department, California Polytechnic State University, San Luis Obispo, CA. Research studies, using ozone in pure water as a direct contact sanitizing agent have been conducted on several agricultural commodities and the results are promising. In the washing of broccoli with water containing up to 1 ppm dissolved ozone, the contact time (CT) necessary for a one-log-fold reduction in aerobic plate count microorganisms was 6.0 minutes. Ozone is an effective germicide and many studies over the years have demonstrated greater lethality rates, however, contact times may be too excessive for some fast-paced industrial operations. Relationships between lethality rate and higher ozone concentrations, or combining ozone with other germicidal processes or pro-oxidants have not yet been conducted. Ozone does not leave a chemical residual and for some industrial sanitizing operations this may be seen as a disadvantage. But, when it comes to our food supply, no residual, and fewer residual by-products is a distinct advantage.

Materials and Methods: For most tests, the commodities were obtained directly from commercial packing houses. In some cases the commodities were personally collected directly from the transport gondola or bin at the processing facility. In a few cases during initial germicidal efficacy tests, the commodities were purchased from a local grocery store and in a few instances, the commodities were collected from the Cal Poly farm. In all, broccoli, broccoflower (a broccoli and cauliflower hybrid), carrots, onions, and tomatoes have been tested. Tomatoes were of the variety called "Jackie". These tomatoes are harvested green and immature subsequently undergoing accelerated ripening and distribution to fresh market. In other words,

all commodities are fresh-market produce not intended for further processing. Approximately 30 to 60 lbs of produce was utilized per experiment, depending on the number of analyses to be performed.

Approximately 3 kg (7 lbs) of the test commodity is placed in a presanitized polypropylene mesh bag and washed in the flume for 3 minutes with no ozone. Another sample was collected as a negative control (raw; no water or ozone) and after laboratory preparation of these control samples the water in the 200 gal test system was charged with ozone to a level of 0.75 to 1.0 ppm (mg/L). Samples then were treated in the water flume for 1, 3 and 10 minutes. Ozone concentration was monitored indirectly using an ORP probe and periodically the ozone concentration in the flume water was determined using the Indigo Method (AccuVac Ampules; HACH Corp.). Microbiological analyses were performed immediately after the wash treatments in the on-site laboratory.

Microbiological examinations included both aerobic count plates and coliform. count plates, American Public Health Association and FDA procedures were followed for all tests and 3M Petrifilm was used as the plating medium.

The ozone test system was constructed to mimic the turbulence found in an industrial flume wash for fruit or vegetables. The system has a 125 gallon reservoir with a one horsepower centrifugal pump capable of moving 40 gallons per minute over the 12 by 1 inch weir. Commodities are immersed in the lower of the two reactor vessels where turbulence is at its greatest.

Results and Discussion: The results of washing experiments depicted in Figure 2.5.12 demonstrate CT values of 9.6 minutes per log-fold reduction in aerobic microbial load for carrots, 7.5 minutes per log-fold reduction for broccoflower, and 6.0 minutes per log-fold reduction for broccoli. For all CT values, the ozone concentration is standardized at 1 ppm.. Six minutes may be too long for a fast-paced industrial wash process. Consequently, the flume water may require an ozone concentration of up to 2 ppm, thus reducing the time factor in half for an equivalent microbial kill. As evident from the data, every commodity is unique and will require a specific treatment to achieve a reasonable reduction in indigenous microbial load.

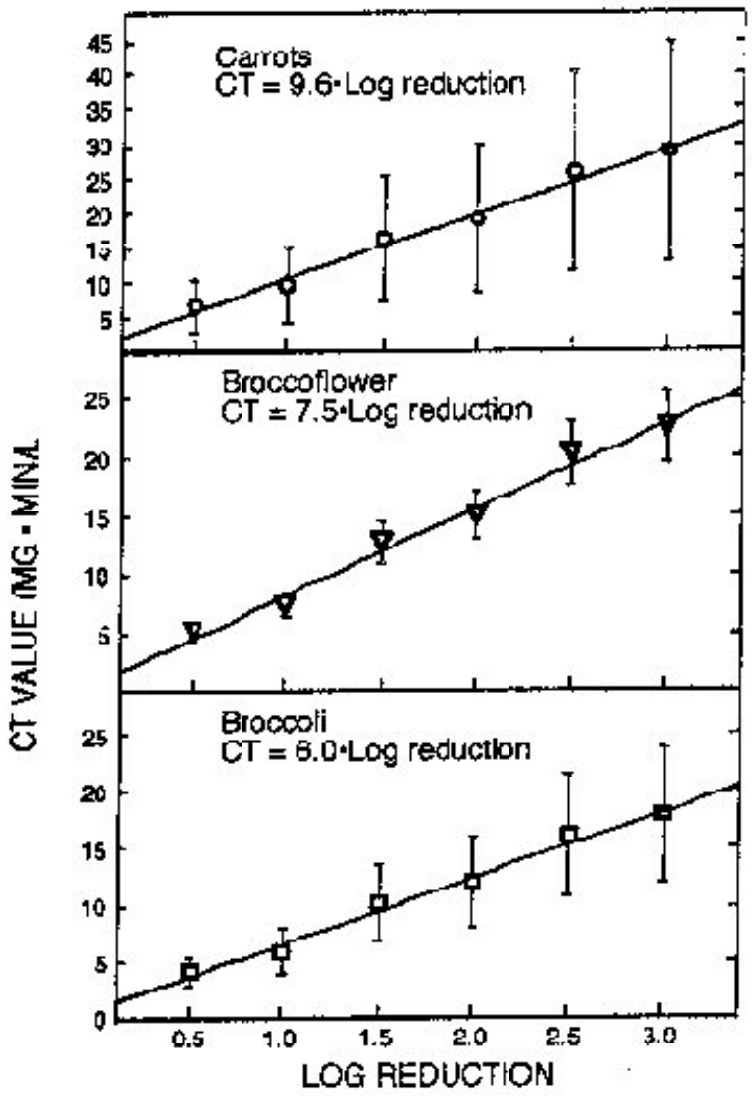


Figure 2.5.12. CT values as time in minutes per log reduction for carrots, broccoflower and broccoli. Values reported were determined by averaging 5, 2, and 11 experiments, respectively (Hampson and Fiori, 1997).

There are numerous variables involved in the washing of produce. Ozone concentration and organic load were controlled in these experiments as was the time of exposure. Other than those factors, variables evident in a flume wash system might include the temperature of the water, the hardness and general chemistry of the water, flow rate in the flume and the contact system, surface area of the commodity, non-target demand substances, types and load of microorganisms present on the commodity, and other variables. Industrial wash systems also may employ surfactants and calcium salts which may or may not have an effect on the CT values achieved.

Every food processing facility is unique and many of these facilities will be able to adapt or retrofit their system to the use of ozone, removing the chlorine which is now in widespread use. If a system is to be retrofitted, an evaluation must first be performed to be sure that safety issues

all are addressed and that there are compatible materials used in the construction of the wash water system. In many cases, it may be advantageous to add a contact reservoir and some type of filtration apparatus to improve on ozone dissolution and contact, and keep the level of non-target demand substances to a minimum. Some produce enters the processing facility with a high organic load (carried in from harvest) and multiple stage wash systems may be the only way to achieve a kill of the microbes with the available ozone in a reasonable period of time. In some systems, such as for tomatoes, the initial wash is at a temperature of 105EF. The high temperature combined with high organic load makes ozonation of the first wash tank difficult.

Figure 2.5.13 shows the reduction in microbial load as a factor of time of exposure. Data presented is an average of three experiments and error bars represent one standard deviation from the mean. Treatments given to the tomatoes are represented along the x-axis (not to scale). Raw tomatoes are untreated; N3 are washed for three minutes in water with no ozone; 1M, 3M, and 10M represent the time of exposure to wash water containing ozone at a concentration of approximately 1 ppm. The experimental system, in the case of this commodity, may not accurately reflect practices in industry since the fresh-market tomato industry typically uses a multi-stage wash system with the first stage having heated water, as mentioned above. Regardless, it is no surprise that ozone is able to have a germicidal effect on the indigenous microflora of fresh tomatoes. One factor not mentioned thus far would be the contribution ozone would have in reducing cross-contamination of the commodity from one load to the next as they pass through the wash system. By maintaining a reduced microbial load in the wash water, one load of produce, which may have a pathogenic microorganism like *E. coli* or spoilage microorganisms not found on other loads, will not contaminate all the produce passing through the wash system in a given period of time. Thus, water maintenance is a major concern to the food processor and should be reason enough to use ozone as a sanitizing agent for their wash water systems.

2.5.8 Kim et al. (1999) – Lettuce

Abstract: When ozone (1.3 mM) was bubbled for 3 min in a mixture of shredded lettuce and water, counts of mesophilic and psychrotrophic bacteria decreased 1.4 and 1.8 $\log_{10}$ cfu/g, respectively. Counts of these microorganisms on lettuce, from a different batch, decreased 3.9 and 4.6 logs, respectively, during 5 min of ozone treatment. Shredded lettuce was treated with gaseous ozone, or mixed with aqueous solution of ozone (1:20 w/w) with or without bubbles. For effective delivery of ozone, stirring (low and high speed), sonication or stomaching was applied during the ozonation. Washing the lettuce with water only decreased total count on shredded lettuce by 0. 74 - 1.0 log cfu/g. When lettuce in a treatment chamber was flushed with gaseous ozone, the total count decreased 0.85 log cfu/g, but when vacuum was applied before the ozone flush, the total count decreased 0. 96 log cfu/g. Bubbling ozone in water-lettuce mixture while sonicating, high-speed stirring, or before stomaching inactivated 1.4, 1.9 and 1.9 log cfu/g, respectively. Bubbling gaseous ozone in water is the most effective ozonation method. Efficient ozone delivery to microorganisms on lettuce requires a combination of ozone bubbling and high-speed stir.

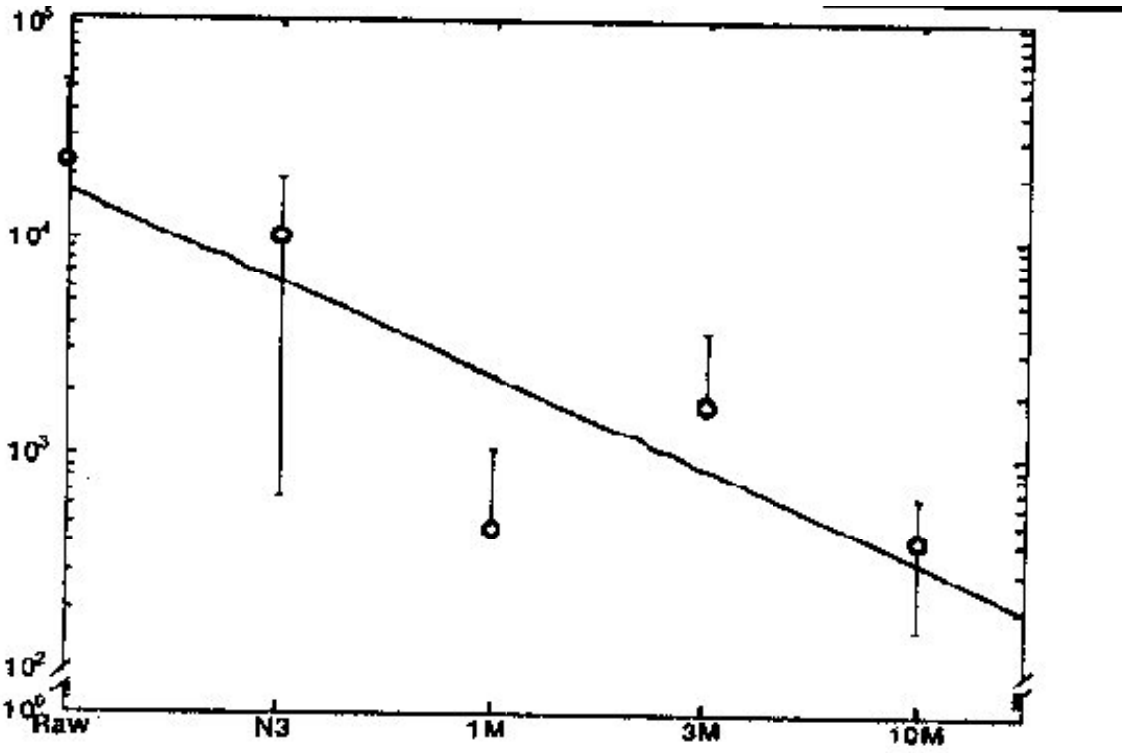


Figure 2.5.13. Germicidal effect of ozone on tomatoes. Raw fruit, washed with non-ozonated water (N3), one minute, three minute, and ten minute washes with water containing ozone at ca 1 ppm (1M, 3M, 10M, resp.) (Hampson and Fiore, 1997).

Materials and Methods

Lettuce: Iceberg lettuce was purchased from the local supermarket and trimmed of discolored and wilted portions. Intact lettuce heads were cut into wedges which were shredded into pieces, 2.5 x 2.5 cm squares, using an electrical knife. Preparation of lettuce was done aseptically.

Treatments to Compare Ozone and Chlorine: Samples (25 each) of shredded lettuce were weighed in stomacher bags in preparation for the decontamination treatment and microbiological analysis. In experiments using ozone, the gas was delivered into the lettuce-water mixture (1:20 w/w) in the stomacher bag through a porous sparger (pore size: 10 Φm). After the treatment, a neutralizer solution was added and bag contents were homogenized in a Stomacher. The bag contents were diluted serially and dilutions were plated, in duplicates, on plate count agar. Plates were incubated at 37EC for 48 h for the mesophilic count, or at 5EC for 7-10 days for counting psychrotrophs. For chlorine treatments, a hypochlorite solution was mixed with the shredded lettuce (1:20 w/w) into a stomacher bag and the mixture was held for a predetermined time. The treated sample was prepared for analysis and mesophilic and psychrotrophic counts were determined as just described.

Challenge Study:

Inoculum: *Pseudomonas fluorescens*, a common lettuce spoilage bacterium, was inoculated onto the lettuce and inactivation kinetics were studied. *P. fluorescens* was obtained from the

culture collection at the food microbiology laboratories of the Ohio State University and the inoculum was prepared as follows: the stock culture was transferred in nutrient broth which was incubated at 26EC for 24 h; this was followed by three successive transfers. After the last incubation, cells were harvested by centrifugation at 3,000 x g in a refrigerated (4EC) centrifuge, washed twice in 0.1 M phosphate buffer solution (pH 7) and suspended, in the same buffer, to a final concentration of 1 x 10^9 to 3 x 10^9 cfu/mL. Suspensions were stirred using a glass rod and a mechanical stirrer to minimize cell clumping. The inoculum size was estimated by measuring A_{600} and calculating approximate count from the standard curve for absorbance versus bacterial count.

Inoculation: Inoculation solution was prepared by mixing 5 mL cell suspension with 495 mL ozone demand-free deionized water in a sterile 1-L beaker. Preliminary experiments were conducted to determine the cell density of the suspension which is sufficient to give an initial population of ~10^4 CFU/g of lettuce. Shredded lettuce was washed with sterile deionized water (1:20 w/w) and spun, using a vegetable spinner, to remove excess water. Washed lettuce was dipped in the inoculation solution, spun and packaged aerobically in a stomacher bag [65 Φm (2.6 mm) thickness]. Some of the shredded lettuce was packaged under a modified atmosphere (20% CO_2, 80% N_2) using a vacuum packaging machine, the inoculum was injected through a septum, and package contents were shaken to distribute the microorganism on the lettuce surface evenly. Packaging material, in this case, was S50d Cryovac (polyvinylidene chloride) bags. Inoculated lettuce samples (packaged aerobically or under modified atmosphere) were stored in the refrigerator (~4EC) for 24 h to allow attachment of cells on the lettuce surface before the treatment with ozone.

Ozonation of Lettuce: Water containing different ozone concentrations (3-10 ppm) was prepared. Ozonated water and inoculated lettuce (ca. 25 g) were mixed in a beaker and stirred gently (using a stirring glass rod) for one minute. The mixture was transferred to a stomacher bag, prepared for microbiological testing, and total microbial count (i.e., natural contaminants plus *Pseudomonas*) was determined as indicated earlier.

Ozonation and Delivery Methods

Ozone Contact System: Uninoculated lettuce was treated with ozone in the gaseous, aqueous or bubbles-containing aqueous states. Delivery of ozone during the treatment was facilitated by stirring, sonication or stomaching. A 600-mL beaker and a 1000-mL stainless steel chamber were used for aqueous and gaseous treatments of lettuce, respectively. Ozone (2 mM or 4.93% v/v) was fed at 0.5 L/min. In case of aqueous treatments, shredded lettuce was mixed with water, ozonated water, or water bubbled with ozone (1:20 w/w dilution) and treated by low-speed (200 rpm) stirring, sonication during low-speed stirring, low-speed stirring followed by stomaching for 2 min, or high-speed stirring (300 rpm). After 5 min of treatment, the lettuce was separated from the aqueous phase by spinning. Untreated and water-washed lettuce, prepared under conditions similar to the ozonation. treatment, was used as a control.

Unwashed or water-washed lettuce was treated with gaseous ozone for 5 min in the gaseous treatment chamber. In some trials, the reaction chamber containing unwashed lettuce was vacuumed and then filled with gaseous ozone.

Microbiological Test: Treated or untreated sample (25 g) of lettuce was mixed with peptone water (1:10, w/w) in a stomacher bag and homogenized for 2 min in a Stomacher. The mixture was serially diluted and plated, in duplicate, on PCA. Plates were incubated at 37EC for 48 h and colonies were counted.

Statistical Analysis: Two control groups (untreated and water-treated samples) and the treatment group (ozone-treated samples) were included in the statistical analysis. Population inactivated (Log_{10} count of untreated sample -- Log_{10} count of the treated counter part) by the treatments was analyzed using MINITAB statistical program (Minitab Inc., State College, PA). Two-way analysis of variance was performed for the effect of ozonation and delivery method. When treatment factors were significant, Tukey=s range test was used for multiple comparison of means.

Results

Comparison of Ozone and Chlorine: Lettuce was treated with hypochlorite solution for 3 min and the rate of inactivation was measured. Chlorine solution, at 1 mM, inactivated 1.4 log mesophilic and 1.8 log psychrotrophic natural contaminants on fresh lettuce (Figure 2.5.14). At 2 mM chlorine, counts of mesophilic and psychrotrophic microorganisms decreased 2.0 and 2.9 log cfu/g, respectively. Inactivation of natural flora on lettuce by ozone (1.3 mM) increased as the exposure time increased (Figure 2.5.15). This ozone treatment inactivated 1.2 and 1.8 log mesophilic and psychrotrophic microorganisms, respectively, in 3 min. The experiment was repeated at a longer contact time and using a different batch of lettuce, and results were compared with those of the control (Figures 2.5.16 and 2.5.17). As the contact time increased, mesophilic microorganisms on fresh lettuce were inactivated to a greater degree; the count decreased ~4 log after 5 min of exposure to ozone. Psychrotrophic count in lettuce showed similar inactivation kinetics; the ozone treatment decreased the count 4.6 log in 5 min. However, counts of mesophiles and psychrotrophs did not change considerably when oxygen was bubbled in the water-lettuce mixture (control treatment).

Challenge Study: Lettuce was inoculated with *P. fluorescens*, stored aerobically or under a modified atmosphere to allow cell attachment, and treated with ozonated water. Count of *P. fluorescens* decreased as the ozone concentration increased, however, ~10 mg/L ozone inactivated only 80% of the population (Figure 2.5.18). Ozone inactivated *P. fluorescens* at a faster rate when the lettuce was stored under aerobic than modified atmosphere conditions before it was ozone-treated.

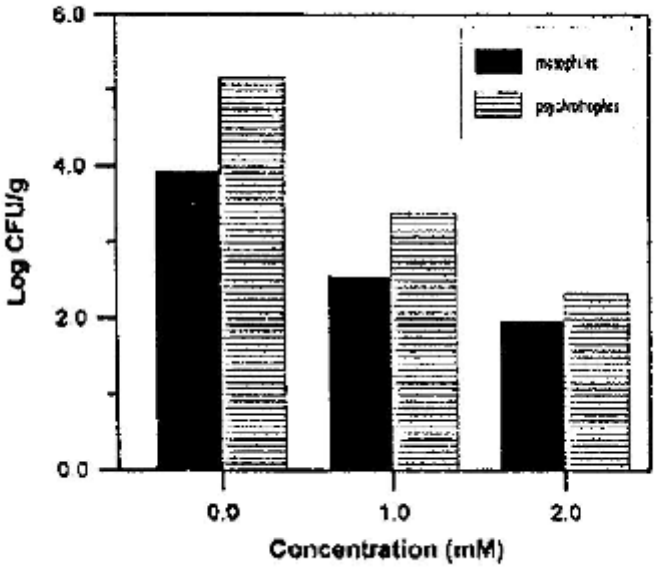


Figure 2.5.14. Counts of natural contaminants on fresh lettuce when treated with chlorine at 25EC (Kim et al., 1999b).

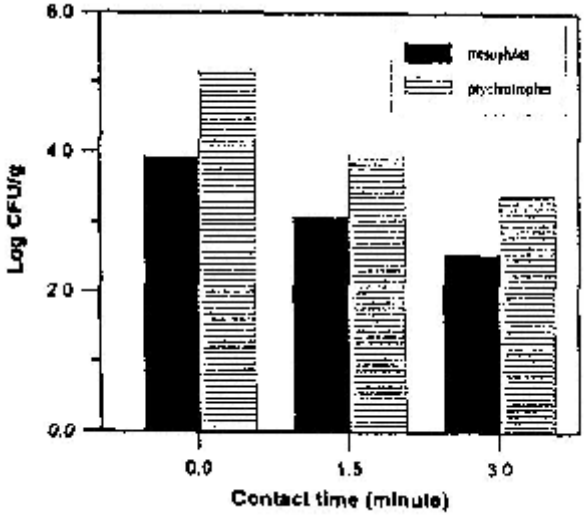


Figure 2.5.15. Counts of natural contaminants on fresh lettuce when treated with 1.3 mM ozone at 25EC (Kim et al., 1999b).

Ozonation and Delivery Method: Lettuce used in these experiments contained 4.5-6.3 log total microflora CFU/g. Water alone, ozonated water and water into which ozone was bubbled removed 0.9, 1.1, and 1.6 log cfu/g, respectively (Figure 2.5.19). Direct ozone bubbling into lettuce-water mixture gave significantly smaller count ($P<0.01$) than water alone. Although

bubbling ozonation gave greater inactivation than did the ozonated water treatment, however, there was no significant difference between these two treatments (P>0.05).

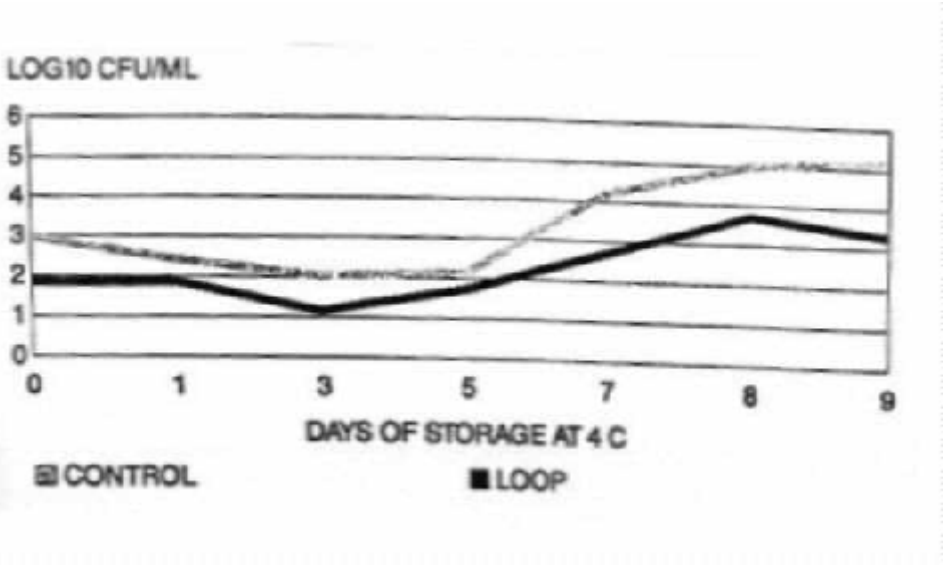


Figure 2.5.16. Inactivation of mesophilic natural contaminants on lettuce by oxygen (control) and ozone (1.3mM) treatment. Count of untreated sample (N_0), count of treated sample (N), ozone (!), oxygen (") (Kim et al., 1999b).

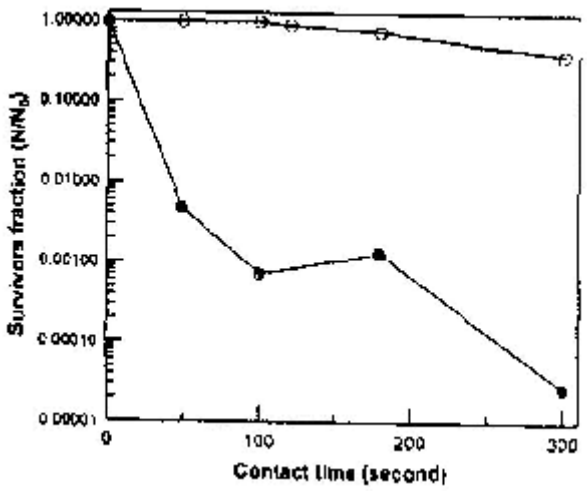


Figure 2.5.17. Inactivation of psychrotrophic natural contaminants on lettuce by oxygen (control) and ozone (1.3mM) treatment. Count of untreated sample (N_0), count of treated sample (N), ozone (!), oxygen (") (Kim et al., 1999b).

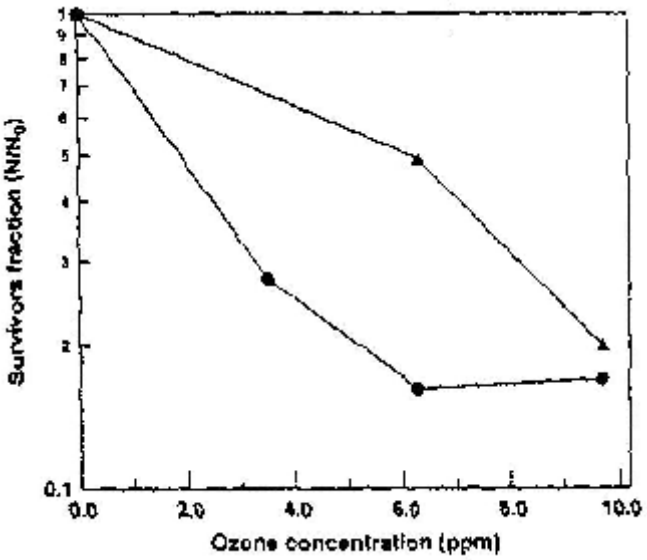


Figure 2.5.18. Inactivation of *Pseudomonas fluorescens* by ozone when inoculated lettuce was stored for 24 h at 4EC before the treatment. Count of untreated sample (N_0), count of treated sample (N), lettuce stored in atmospheric packaging (!), lettuce stored under modified atmosphere packaging ( ) (Kim et al., 1999b).

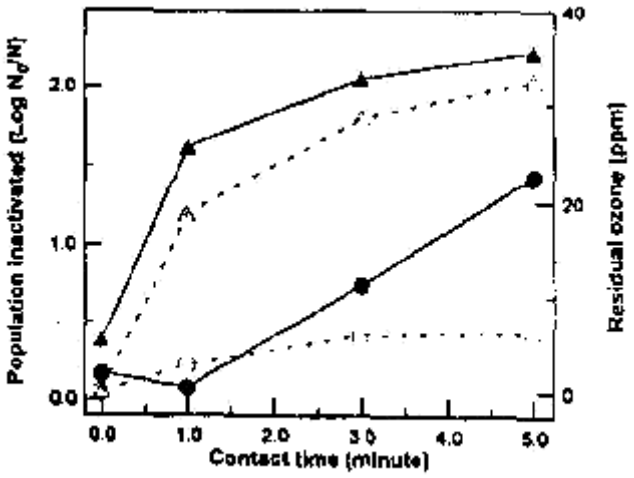


Figure 2.5.19. Inactivation of natural contaminants on fresh lettuce by different ozonation methods. Means and standard errors are shown. Different letter indicates significant difference at $p<0.01$. Count of untreated sample (N_0), count of treated sample (N) (Kim et al., 1999b).

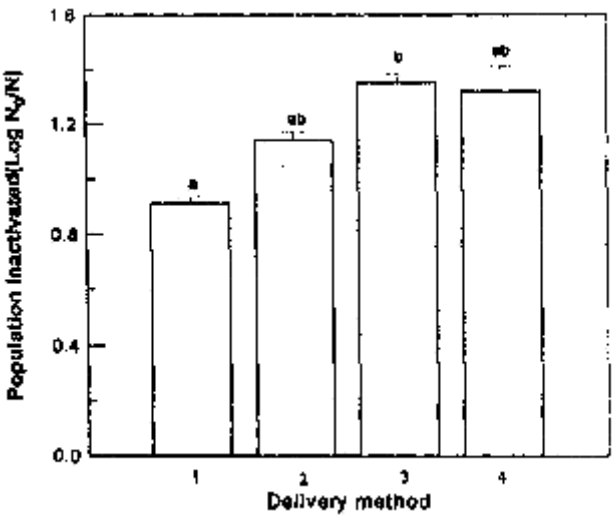


Figure 2.5.20. Inactivation of natural contaminants on fresh lettuce by ozonation plus different agitation methods. 1: low-speed stir; 2: low-speed plus sonication; 3: low-speed stir followed by stomaching; 4: high-speed stir. Count of untreated sample (N_0), count of treated sample (N). Means and standard errors are shown. Different letter indicates significant difference at $p<0.05$ (Kim et al., 1999b).

Inactivation of natural contaminants on lettuce by ozone varied with the delivery method. Population of natural microflora on lettuce decreased 0.9 log for low-speed stir, 1.1 for low-speed stir plus sonication, 1.4 for low-speed stir and stomaching and 1.3 for high-speed stir (Figure 2.5.20). A significant difference ($P<0.05$) in microbial inactivation between low-speed stir and low-speed stir plus stomaching was detected.

To compare combinations of ozonation and delivery methods, one way of analysis of variance was performed using combined data (Table 2.5.08). The largest inactivation of natural contaminants on lettuce was obtained when bubbling ozone treatment was combined with stir plus stomaching or high-speed stir; the decrease in population in both cases was 1.9 log cfu/g ($P<0.05$). The least reduction was obtained when water without ozone was used for the lettuce treatment at low-speed stir; average decrease in count was 0.74-log only.

Bubbling ozone with high-speed stir seems the most efficient and applicable way of ozone treatment for lettuce processing. Therefore, bubbling ozone treatment was run at different contact time and the residual ozone concentration was monitored during the treatment (Figure 2.5.21). Initial microbial load on lettuce was 3.8, and 4.9 log cfu/g for the high- and low-speed stir treatments, respectively. When the exposure time increased, inactivation rate by both treatments also increased. At high-speed stir, the inactivation proceeded faster than in low-speed stir. More than 2-log contaminants on lettuce were inactivated during 5 min of ozonation. The rate of inactivation during high-speed stir treatment diminished after 3 min, while in low-speed stir treatment, it increased progressively in 5 min. The rate of ozonation (as indicated by the amount of residual ozone) in lettuce-water mixture was smaller when low- instead of high-speed stir was used. Residual ozone was 33 and 7 ppm after 5 min of ozonation which was accompanied with high- and low-speed stir, respectively. High speed agitation may have helped

incorporate greater amounts of ozone in the reaction mixture and thus a greater amount of residual ozone remained in the mixture. Additionally, differences in microbial loads and other ozone-demand materials on lettuce used in these experiments may have contributed to these variations in residual ozone.

Table 2.5.08. Inactivation of natural contaminants on fresh lettuce by combined ozonation and delivery method (Kim et al., 1999b)

Delivery Method	Ozonation Method		
	Water	Ozonated Water	Bubbling Ozone
Low-speed stir	$0.74^{a} \forall 0.06$	$0.98^{a} \forall 0.12$	$1.01^{a1} \forall 0.05$
Low-speed stir + Sonication	$1.02^{a} \forall 0.10$	$0.98^{a} \forall 0.10$	$1.43^{b1} \forall 0.07$
Low-speed stir + Stomaching	$0.84^{a} \forall 0.16$	$1.28^{a} \forall 0.07$	$1.93^{b2} \forall 0.10$
High-speed stir	$1.03^{a} \forall 0.49$	$1.05^{a} \forall 0.49$	$1.88^{b1} \forall 0.16$

* Data are means of population inactivated ∀ standard error.

** Means with different superscripts in a same row or column are significantly different ($P<0.05$).

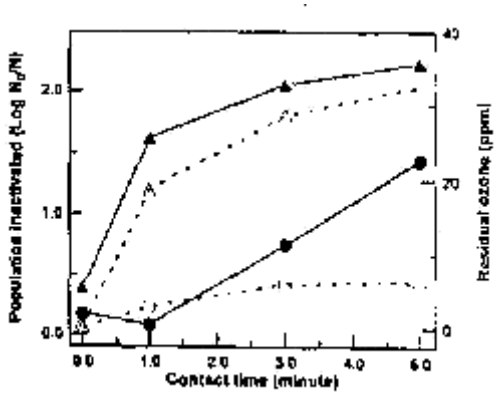


Figure 2.5.21. Inactivation of natural contaminants on lettuce by ozone at different stir speeds (200 and 300 rpm), and associated changes in residual ozone. Count of untreated sample (N_0), count of treated sample (N), residual ozone at 200 rpm ("), residual ozone at 300 rpm ()), population inactivated at 200 rpm (!), population inactivated at 300 rpm ( ) (Kim et al., 1999b).

The decrease in natural contaminants on lettuce was 0.50 log and 0.85 log for water washed and unwashed lettuce, respectively, which were treated with gaseous ozone. The count decreased 0.96 log when the treatment chamber, containing unwashed lettuce, was vacuumed and flushed with ozone. Therefore, gaseous ozone treatment was less effective than ozonated water and bubbling ozone treatment in terms of microbial inactivation.

Discussion

Bubbling ozone (1.3 mM) in water-lettuce mixture for 3 min inactivated ~2-logs of natural flora (Figure 2.5.15). When duration of the ozonation treatment was extended to 5 min, populations of mesophilic and psychrotrophic microorganisms decreased 3.9 and 4.6 logs, respectively (Figures 2.5.16 and 2.5.17). This long exposure time, however, is likely to be impractical in food applications.

Challenge Study: Lettuce was inoculated with *P. fluorescens*, refrigerated for 24 h to allow different degrees of cell attachment, and immersed in water containing ~3 to 10 ppm ozone. The ozone dip decreased the count of *Pseudomonas* <1-log on lettuce which was refrigerated overnight before the treatment (Figure 2.5.18). Dipping the lettuce in ozone water with minimal agitation and attachment of cells to lettuce surface most likely contributed to this modest decrease in count. Therefore, bubbling is probably more efficient than dipping as a method to deliver ozone to the treated product.

Ozonation and Delivery Method

Since direct ozone bubbling is more efficient than ozonated water for decontaminating lettuce, combinations of ozonation methods with different degrees of turbulence were tested.

Water Rinsing: The effective dose of a disinfectant (e.g., ozone) depends greatly on water quality (i.e., temperature, pH, salt content, and organic and microbial load). When water is recycled during vegetable processing, it likely accumulates debris and microbial contaminants. Therefore, ozone treatment may become inefficient when applied at a conventional vegetable processing environment. In this study, water washing removed only 0.85-log of contaminants from the lettuce (Figure 2.5.19).

Degree of Agitation: Lettuce-water mixture was ozonated at different stirring speeds, sonication and stomaching. High-speed stir during ozonation enhanced appreciably the ability of ozone to inactivate microorganisms on lettuce (Table 2.5.08 and Figure 2.5.20). Sonication and stomaching also improved the ozone disinfection rate as compared to low-speed stir. Decomposition of ozone is rapid in the water phase of foods, such that its antimicrobial action may take place mainly at the surface. Most of the microorganisms may not be found in free suspension as discrete particles, especially in food systems. The association of organisms or with suspended matter may hamper the accessibility of ozone to microorganisms.

Inactivation of microbial contaminants on lettuce was enhanced when ozone was bubbled in ozonated water-lettuce mixture (Table 2.5.08 and Figure 2.5.19). Bubbling ozone gas in water with a rapid stirring provided the best ozone delivery system for the inactivation of microorganisms on lettuce. Inactivation of microflora on lettuce was greatest (1.86 to 1.91 log cfu/g) when ozone bubbling was applied during high-speed stir or with stomaching. Inactivation rates of microorganisms are closely related to residual ozone in the treated mixture. For greater inactivation by gaseous ozone, reactor designs need to be improved.

2.5.9 Anonymous Celery Processor (1999)

This information was submitted to the EPRI Food and Agriculture Alliance under conditions of anonymity pending completion of the on-going study program.

Ozonated water (0.9 ppm ozone at 60 sec contact time) is used to clean chopped celery. The tests are well designed, with sufficient samples to provide statistical analysis (average and standard deviation calculated for all tests). At 0.9 ppm ozone in rinse water, log of count reduction for total aerobic plate count (TAPC), coliform, psychrophiles, yeast and molds is 1.0 to 1.3:

TAPC	0.9 log
Coliforms	3.0 logs
Psychrotrophs	0.86 log
Yeast and Mold	0.66 log

BOD load in the rinse water, from celery juice released by chopping, is high 26,700 + 5800 mg/kg before and 20,840 + 3,700 mg/kg after treatment with 1.2 ppm ozone and 60 sec contact time (22% reduction):

TAPC	from 0.91 to 2.1 log = 1.2 logs
Coliforms	from 2.0 to 3.00 logs = 1.0 log
Psychrotrophs	from 0.67 to 2.1 log = 1.25 logs

The ozone results compare favorably with Tsunami 40-50 ppm.

SUMMARY OF VEGETABLES SECTION

Numerous independent investigations have documented the efficacy and safety of aqueous ozone washes and gaseous ozone treatment for reducing microbial counts on a wide variety of vegetable products. Microbial studies typically show 2-logs or greater reduction of total counts and significant reduction of specific spoilage and potentially pathogenic species most commonly associated with vegetable products. Lethality of ozone is greatest against microorganisms suspended in water. Microorganisms embedded in product surfaces are more resistant. Applications methods must assure direct contact of ozone with the target microbial cells. A variety of methods have been used to accomplish this, including stirring, pumping, fluming, bubbling, sonication, abrasion, pressure washing, etc. Gaseous ozone similarly is effective against many species of contaminants, but it is more difficult to enssure contact of gaseous ozone with the target microbial cells.

Thiobarbituric acid (TBA) values, vitamin C determinations, colorimetric analyses, air quality monitoring, and sensory observations ensure that effective ozone treatment is not harmful to the processed food products.

2.6 MISCELLANEOUS APPLICATIONS

2.6.1 Flours

2.6.1.1 Naito et al. (1987a)

This article is in Japanese with an English abstract. However, tables and figures with meaningful data are in English and are included as appropriate.

Abstract: In order to study the food preservative effect of ozone, the microbiocidal effect of ozone was investigated employing cereal grains, cereal grain powders (flours), peas, beans and whole spices. The conditions employed to evaluate the microbiocidal effect were: ozone gas concentration 0.5 ~ 50 ppm, temperature 5 ~ 50EC, time 1 ~ 6 hour, and flow rate 100 L/min.. Results were as follows: (1) The microbiocidal effect of ozone was affected by contact concentration. Higher contact concentration resulted in greater microbiocidal effect on various cereal grain powders (flours). When buckwheat whole grain with hull (Japan), black matpe and black pepper (seed) were treated with 5.0 ppm of ozone, the microbiocidal effect of ozone was maximum. (2) Whole, halves, and ground samples of buckwheat were treated with 50 ppm of ozone continuously for 1 hour at 10EC. The microbiocidal effect of ozone was maximum in whole and minimum in ground. (3) Lower temperature resulted in greater microbiocidal effect on cereal grains, cereal grain powders (flours), peas, beans and whole spices except black pepper. (4) Longer contact time resulted in greater microbiocidal effects on cereal grains, cereal grain powders, peas, beans and whole spices, except buckwheat whole grain with hull and black rnatpe.

Table 2.6.01 shows the number of microorganisms normally found in various food ingredients (including grain powders = flours) used for food manufacture. Figure 2.6.01 shows the effect of ozone concentration on the pasteurization efficiency in various food ingredients. Figure 2.6.02 shows the effect of ozone temperature on the pasteurization efficiency in various food ingredients. Figure 2.6.03 shows the effect of ozone treatment time on the pasteurization efficiency in various food ingredients. Figure 2.6.04 shows the effect of ozone treatment time on the pasteurization efficiency in Japanese buckwheat flour.

2.6.1.2 Naito et al. (1989c) -- Japanese Raw Noodles)

This article is in Japanese with an English abstract. However, tables and figures with meaningful data are in English and are included as appropriate.

Abstract: This study was carried out to see the extent to which ozone treatment of flour controls the growth of airborne microorganisms in a factory and the degree to which ozone treated flour improves the shelf life of "namamen" Japanese Raw Noodles). The growth of microorganisms in "namamen" prepared from ozone treated wheat flour was remarkably inhibited. The storage life of packaged "namamen" prepared from wheat flour treated with ozone at 0.5 ~ 50 ppm for 6 hours became 2~5 times longer. Some thiamin decomposition was detected in "namamen" prepared from wheat flour treated with ozone at 0.5 ~ 50ppm for 6 hours. When the "namamen" was stored at 51EC for 30 days, the thiamin content decreased from 87~97% to 70~93%. There was no significant change in the riboflavin content in "namamen" prepared from wheat flour

treated with ozone at 0.5 ~ 50ppm for 6 hours. Changes in quality of "namamen" prepared from wheat flour treated with ozone at 0.5 ~ 5.0 ppm for 6 hours were not observed in appearance, odor, taste, texture and overall evaluation.

Table 2.6.01. Number of microorganisms in food ingredients used for food manufacture (Naito et al., 1987a)

Food Ingredient	Number of microorganisms					
	Total microorganisms		Heat-resistant bacteria			
			Mesophiles		Thermophiles	
	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum
Whole grains	2.5×10^5	3.0×10^4	8.5×10^3	3.5×10^2	7.5×10^3	1.5×10^2
Grain powders (flours)	8.1×10^5	5.0×10^3	4.5×10^3	1.5×10^2	7.0×10^2	2.1×10
Peas and beans	5.0×10^4	1.5×10^3	3.5×10^3	1.5×10^2	1.0×10^2	3.0×10
Whole spices	3.1×10^5	1.0×10^3	7.5×10^4	3.1×10^3	2.0×10^3	1.5×10^2

Table 2.6.02 shows the number of survivors in microflora of wheat flour treated with ozone, packaged in a polyethylene pouch and stored at 10EC and 30EC for 10 days. Table 2.6.03 shows the changes in airborne organisms at various steps in namamen processing. Table 2.6.04 shows the change in microbial counts with the ingredients (wheat flour, salt and water) at various stages during namamen processing. Figure 2.6.05 shows the growth in bacteria in namamen prepared from ozone-treated (0-50 ppm) wheat flour and stored at 20EC. Figure 2.6.06 shows the growth of yeast in namamen prepared from ozone-treated (0-50 ppm) wheat flour and stored at 20EC. Figure 2.6.07shows the growth of fungi prepared from ozone-treated (0-50 ppm) wheat flour and stored at 20EC.

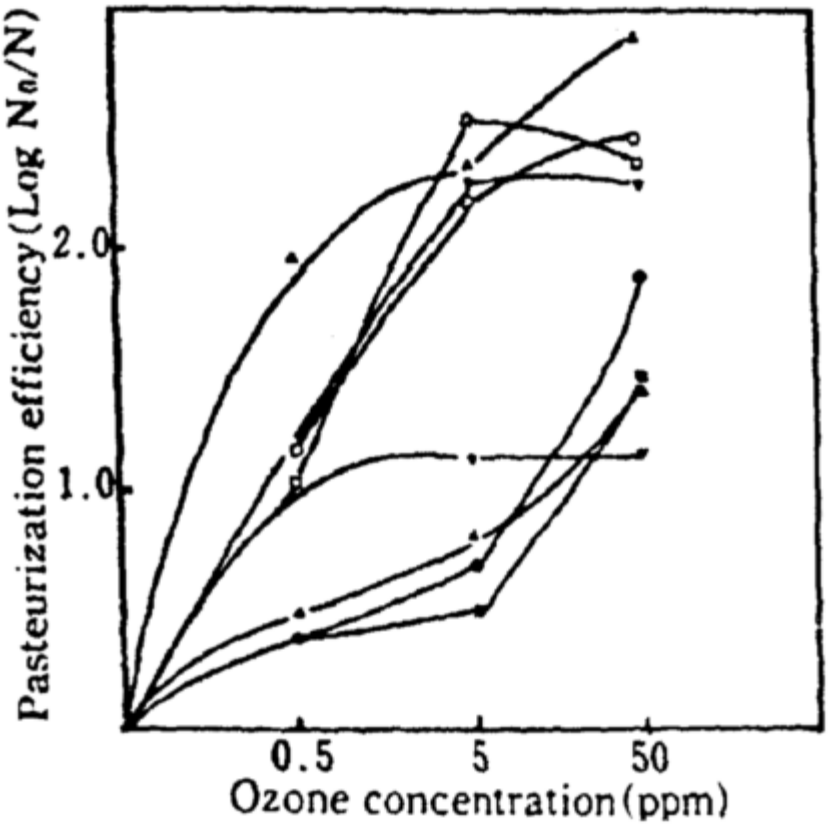


Figure 2.6.01. Effect of ozone concentration on the pasteurization efficiency in various food ingredients: " whole grains (max);) grain powders (flours) (max); G peas and beans (max); Λ whole spices (max); ! whole grains (min); grain powders (flours) (min); # peas and beans (min); whole spices (min). N_0 and N indicate the initial numbers of microorganisms and survivors after ozone treatment, respectively. Max and Min indicate the maximum and minimum pasteurization efficiency of initial contaminating microorganisms in each group of tested food ingredients. Ozone treatment period: 1 hour; ozone treatment temperature: 20EC (Naito et al., 1987a).

2.6.2 Spices

2.6.2.1 Zhao and Cranston (1995) – Whole Black Peppercorn

Abstract: The use of ozone as a substitute for ethylene oxide to decontaminate whole black peppercorn and ground black pepper and the effects of ozone on the volatile oil constituents of the spice were studied. Black peppercorns were immersed in water and sparged with ozone-containing air (ozone concentration, 6.7 mg liter^{-1}) for 10 min at an air flow rate of 6 liter min^{-1}. This treatment reduced the microbial population of peppercorn by 3-4 log numbers. Ground black pepper with various moisture levels was sparged with ozone-containing air for up to 6 h. This treatment reduced the microbial population by 3-6 log numbers, depending on the moisture content of the spice. Higher moisture content led to a greater reduction in microbial load. The

volatile oil constituents of the spice with and without ozone treatment were extracted with isopropyl ether and analyzed by gas chromatography and gas chromatography-mass spectrometry. Ozone treatment of ground black pepper resulted in the oxidation of certain volatile oil constituents while the treatment had no significant effect on the volatile oil constituents of whole peppercorn.

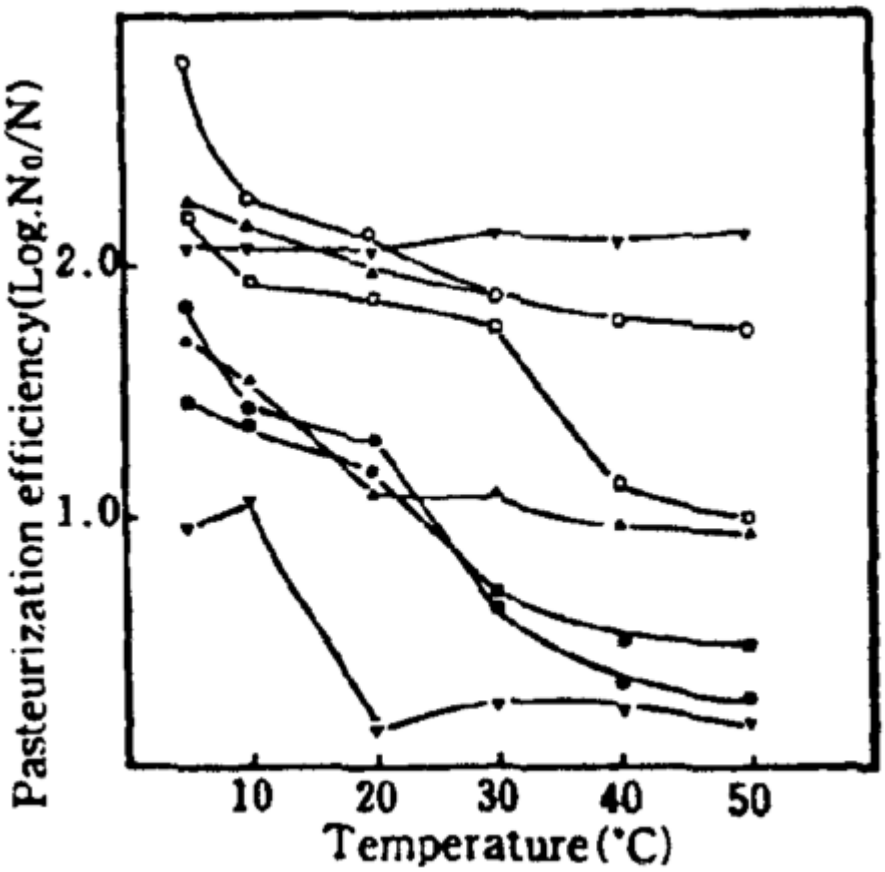


Figure 2.6.02. Effect of ozone treatment temperature on the pasteurization efficiency in various food ingredients: " whole grains (max);) grain powders (flours) (max); G peas and beans (max); Λ whole spices (max); ! whole grains (min); grain powders (flours) (min); # peas and beans (min); whole spices (min). Ozone treatment period: 1 hour; ozone concentration: 50 ppm (Naito et al., 1987a).

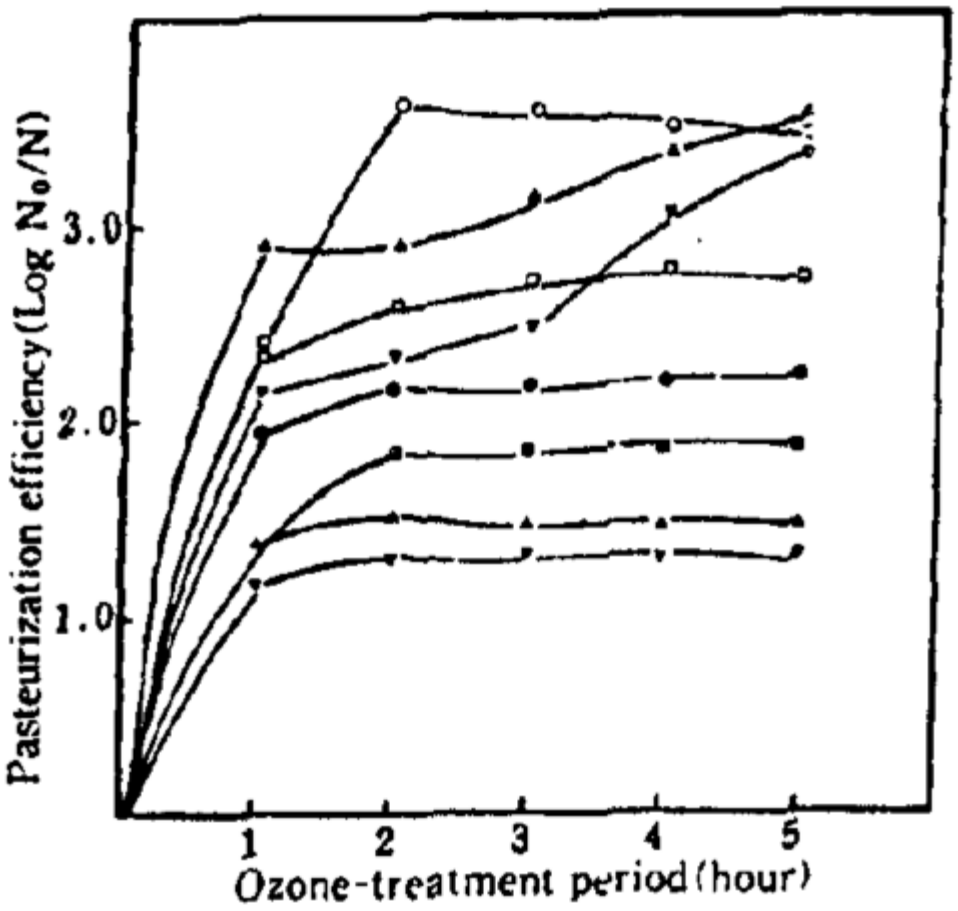


Figure 2.6.03. Effect of ozone treatment time on the pasteurization efficiency in various food ingredients: " whole grains (max);) grain powders (flours) (max); G peas and beans (max); Λ whole spices (max); ! whole grains (min); grain powders (flours) (min); # peas and beans (min); whole spices (min). Ozone treatment temperature: 20EC; ozone concentration: 50 ppm (Naito et al., 1987a).

Experimental

A flow rate of 6 liters min^{-1} was maintained through the ozone generator (ozone generated from dried air) for all experiments. At this flow rate, the concentration of ozone in the air was 6.7 mg $liter^{-1}$.

Determination of Moisture Content of Black Pepper: The moisture content of ground black pepper was determined by the air-oven method and expressed as g kg^{-1} in all cases.

Growth of Microbial Cultures: Cultures of *Escherichia coli*, *Salmonella* spp, *Staphylococcus aureus* and *Bacillus cereus*, previously stored on nutrient agar slants, were subcultured and grown on nutrient agar at 30EC for 24 h. Cultures of *Penicillium* spp and *Aspergillus* spp, previously stored on malt extract agar slants, were subcultured and grown on malt extract agar at

25EC for 3 days. Each culture was washed with sterile peptone water solution (1.0 g kg^{-1}), resulting in suspensions with cell concentrations of approximately 10^{11} - 10^{12} cfu $liter^{-1}$. Ground black pepper was inoculated with the cell suspensions so that it contained approximately 10^{10} to 10^{11} cfu kg^{-1} spice. Aliquots of the cell suspensions were treated with ozone-containing air to study the resistance of different microorganisms to ozone in aqueous solution.

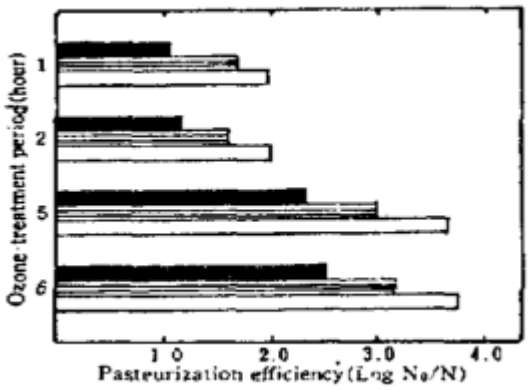


Figure 2.6.04. Effect of ozone treatment time on the pasteurization efficiency in Japanese buckwheat flour. Ozone concentration (ppm): # 0.5; ɔ 5; G 50; ozone treatment temperature 10EC (Naito et al., 1987a).

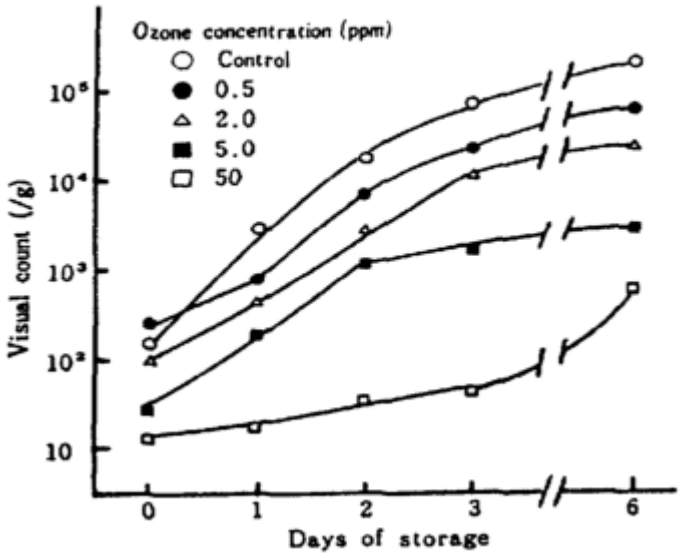


Figure 2.6.05. Growth of bacteria in namamen prepared from ozone-treated wheat flour and stored at 20EC (Naito et al., 1989c).

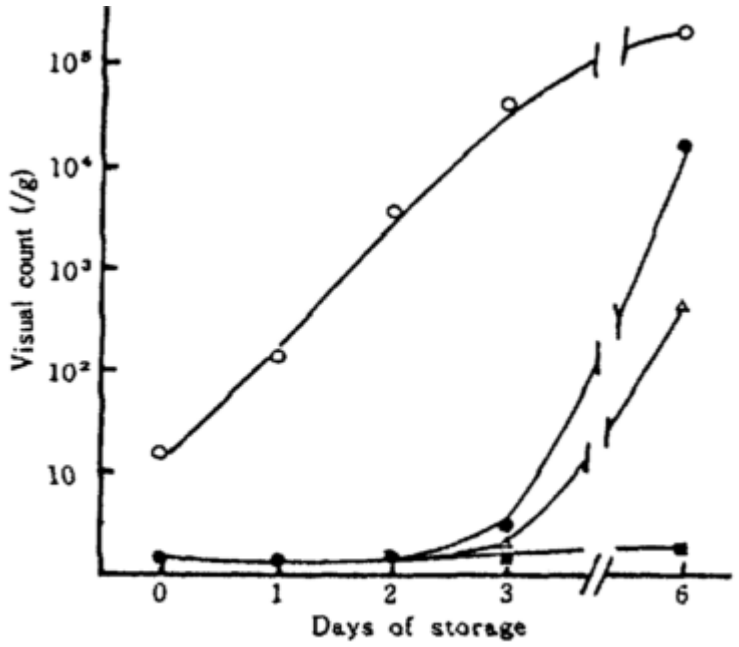


Figure 2.6.06. Growth of yeast in namamen prepared from ozone-treated wheat flour and stored at 20EC. Ozone concentrations (ppm): " Control; ! 0.5,) 2.0; # 5.0; Q 50 (Naito et al., 1989c).

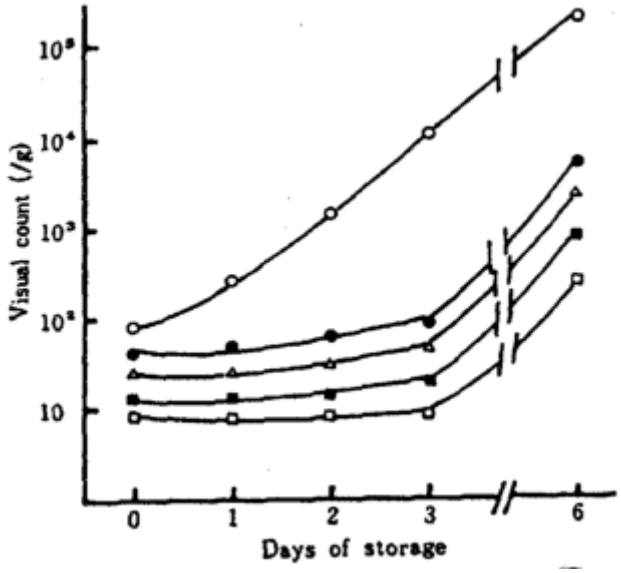


Figure 2.6.07. Growth of fungi in namamen prepared from ozone-treated wheat flour and stored at 20EC. Ozone concentrations (ppm): " Control; ! 0.5,) 2.0; # 5.0; Q 50 (Naito et al., 1989c).

Table 2.6.02. Number of survivors in microflora of wheat flour treated with ozone, packaged in a polyethylene pouch and stored at 10E, 30EC for 10 days (Naito et al., 1989c)

	Viable Counts (/g)			
	Ozone Concentration (ppm)			
	0.5	**2.0**	**5.0**	**50**
Total Survivors				
Before storage	1.1×10^3	12.3×10^3	1.4×10^3	1.0 x
10EC storage	8.1×10^2	7.6×10^2	1.3×10^2	7.5 x 10
30EC storage	2.0×10^2	1.5×10^2	6.5 x 10	5.5 x 10
Mesophilic Heat-Resistant Bacteria				
Before storage	5.0 x 10	7.0 x 10	6.0 x 10	3.1 x 10
10EC storage	3.5. x 10	3.0 x 10	3.5 x 10	< 3.0 x 10
30EC storage	3.5 x 10	4.0 x 10	3.5 x 10	< 3.0 x 10
Thermophilic Heat-Resistant Bacteria				
Before storage	3.1 x 10	3.3 x 10	< 3.0 x 10	< 3.0 x 10
10EC storage	< 3.0 x 10	< 3.0 x 10	< 3.0 x 10	< 3.0 x 10
30EC storage	< 3.0 x 10	< 3.0 x 10	< 3.0 x 10	< 3.0 x 10
Fungi				
Before storage	1.5×10^2	1.0×10^2	3.0 x 10	3.5 x 10
10EC storage	8.0 x 10	9.5 x 10	< 3.0 x 10	< 3.0 x 10
30EC storage	6.5 x 10	8.0 x 10	< 3.0 x 10	< 3.0 x 10
The samples were treated with ozone at 5EC for 6 h. Viable counts are expressed as total of bacteria and fungi.				

Application of Ozone to Microbial Cell Suspensions: Ozone-containing air was sparged into each of the cell suspensions (500 mL) contained in glass bottles. The ozone dose was controlled by the exposure time. Immediately before and after the ozone treatment, 10 mL aliquots of the cell suspensions were withdrawn and quantitatively cultured.

Application of Ozone to Ground Black Pepper: Ozone-containing air was sparged into 500 g ground black pepper contained in a round bottom flask (modified from a rotary evaporator). The sparger in the middle of the rotating flask ensured the ozone-containing air was evenly distributed throughout the spice. After the ozone treatment, 50 g of the spice was taken and analyzed microbiologically and for volatile oil constituents.

Table 2.6.03. Changes in airborne microorganisms in namamen processing (Naito et al., 1989c)

Processing	Viable Count							
	Pin Hole Sampler Method				Fallout Method			
	Manufactory		Laboratory		Manufactory		Laboratory	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
Mixing and Agitating	115	25	47	15	88	20	20	15
Folding and Sheeting	105	30	51	12	85	25	22	13
Final Sheeting	110	28	48	16	80	26	18	12
Cutting	115	26	40	18	82	28	25	15
Packaging	118	25	40	18	83	21	20	10
Values of pine hole sampler method are expressed as colony forming units per 53 L air. Values of fallout method are expressed as colony forming units by exposure of the plates for 5 min.								

Washing of Whole Peppercorn with Ozonized Water: Whole peppercorns (200 g) were immersed in 500 mL distilled water in a glass bottle. Ozone-containing air then was sparged into the water-spice mixture for defined periods of time. Samples (50-g) were withdrawn and analyzed microbiologically and for volatile oil constituents.

Microbiological Analysis: Total aerobic bacteria were enumerated by the spread plate method using standard plate count agar, and by incubating plates at 30EC for 48 h. Total anaerobic bacteria were enumerated by the same method except that the plates were incubated in anaerobic jars. Mesophilic aerobic spore formers were enumerated by the pour plate method using tryptone glucose extract agar. *Escherichia coli* was enumerated by the Anderson Baird-Parker quick method. *Staphylococcus aureus* was enumerated by the plate count method using Baird-Parker agar. *Bacillus cereus* was enumerated by the plate count method of Holbrook and Anderson using PEMBA agar. Because there is no reliable method available for enumeration of *Salmonella* spp in a mixed culture, the organism was enumerated by the following method: ground black pepper (200 g) was sterilized by autoclaving and dried at 105EC for 24 h. The sterilized spice then was mixed with a suspension of *Salmonell*a culture in a predetermined proportion so that the spice contained approximately 10^{10} to 10^{11} cfu kg^{-1}. The organisms in the spice before and after ozone treatment were enumerated by the standard plate count procedure. Spores of *Penicillium* spp and *Aspergillus* spp were enumerated by the method using DRBC agar plates, which were incubated at 25EC for 5 days.

Table 2.6.04. Changes in microbial counts in namamen processing (Naito et al., 1989c)

	Viable Counts (/g)			
	Control		Ozone Treatment	
	Manufactory	Laboratory	Manufactory	Laboratory
Ingredients				
Wheat flour	3.5×10^2	3.5×10^2	5.5 x 10	5.5 x 10
Salt	< 3.0 x 10	< 3.0 x 10	< 3.0 x 10	< 3.0 x 10
Water	< 3.0 x 10	< 3.0 x 10	< 3.0 x 10	< 3.0 x 10
Mixing and agitating	8.7×10^2	4.0×10^2	8.0 x 10	6.5 x 10
Folding and sheeting	1.2×10^3	3.8×10^2	7.6 x 10	6.0 x 10
Final sheeting	5.5×10^3	3.5×10^2	8.5 x 10	6.5 x 10
Cutting	7.5×10^3	5.6×10^2	9.0 x 10	7.0 x 10
Packaging	8.0×10^3	3.0×10^2	1.2×10^2	9.5 x 10
Ozone: Wheat flour was treated with 5.0 ppm ozone at 5EC for 6 h. Viable counts are expressed as total of bacteria and fungi.				

Results

Effect of Ozone on Microorganisms in Aqueous Solution: Ozone was found to be effective in reducing the population of several important groups of bacteria and fungi. The data are presented in Table 2.6.05 which gives the time required for reducing the microbial populations in 1.0 g kg^{-1} peptone water solutions by 5 log numbers (99-999% kill), and Figure 2.6.08 which shows the kinetics of the changes. *Staphylococcus aureus* and *Bacillus cereus* exhibited stronger tolerance to ozone than did *E coli* and *Salmonella* spp. The tolerance of the two fungal species to ozone also was different; while an ozone concentration of 0.4 g $liter^{-1}$ was sufficient to reduce the population of *Aspergillus* spp. by five log numbers, 1-8 g $liter^{-1}$ was needed to inflict a similar reduction on the population of *Penicillium* spp.

Effect of Gaseous Ozone Treatment on Microorganisms in Ground Black Pepper: Passing ozone-containing air through ground black pepper reduced the microbial level in the spice. The data are presented in Figure 2.6.09. Populations of Salmonella spp and *E. coli* were reduced by more than 3 log numbers after being treated with ozone-containing air for 60 min at an ozone input of 40 mg min^{-1}. The population of *Penicillium* spp in the spice was reduced by more than 3 log numbers after being treated with ozone-containing air for 40 min, while the population of *Aspergillus* spp was reduced by more than 4 log numbers after being treated for 10 min.

Table 2.6.05. Tolerance of different microorganisms to ozone in peptone water solution (1.0 g kg^{-1})[a] (Zhao and Cranston, 1995)

Microorganism	Concentration of ozone[b] (g $liter^{-1}$)
E. coli	0.40
Salmonella spp	0.40
Staphylococcus aureus	1.20
Bacillus cereus	1.20
Aspergillus spp	0.40
Penicillium spp	1.80

a Results are expressed as the minimum concentration of ozone required to reduce the microbial population by 5 log cycles (99-999% kill).

b The microbial suspensions were sparged with ozone-containing air for defined periods of time. The concentration of ozone in the suspensions was obtained by multiplying the ozone concentration in the air (6-7 mg $liter^{-1}$), the air flow rate (6 liters min^{-1}) and the time of the ozone treatment.

Influence of Moisture Content of Spices on the Effectiveness of Gaseous Ozone Treatment: The effectiveness of ozone on the reduction of microorganisms in ground pepper was strongly influenced by the moisture content of the spice. Higher moisture content led to a greater reduction in the microbial load in the spice. The greatest reduction in microbial population occurred in the ground pepper with a moisture content of 176.0 g kg^{-1} and the least reduction occurred in the spice which had a moisture content of 39.0 g kg^{-1} (Table 2.6.06), but the influence of moisture content on the reduction of total aerobic bacteria and total anaerobic bacteria became evident only after more than 2 h of the ozone treatment (Figure 2.6.10).

The reduction of microbial population in the spice occurred mostly in the initial 2 h of ozone treatment. After that time, the rate of the microbial population reduction slowed down considerably, and for spice with an initial moisture content of 39.0 g kg-1, ozone treatment beyond 2 h did not produce appreciable further reduction in the microbial population (Figure 2.6.10).

Washing Whole Peppercorn with Ozone-Containing Water: When ozone was pumped through a suspension of whole black peppercorns in distilled water for various times, the microbial load on the peppercorn decreased (Figure 2.6.11). Total aerobic bacteria, total anaerobic bacteria and mesophilic aerobic spore formers each were reduced by 3-4 log numbers after being treated with ozone for 10 min.

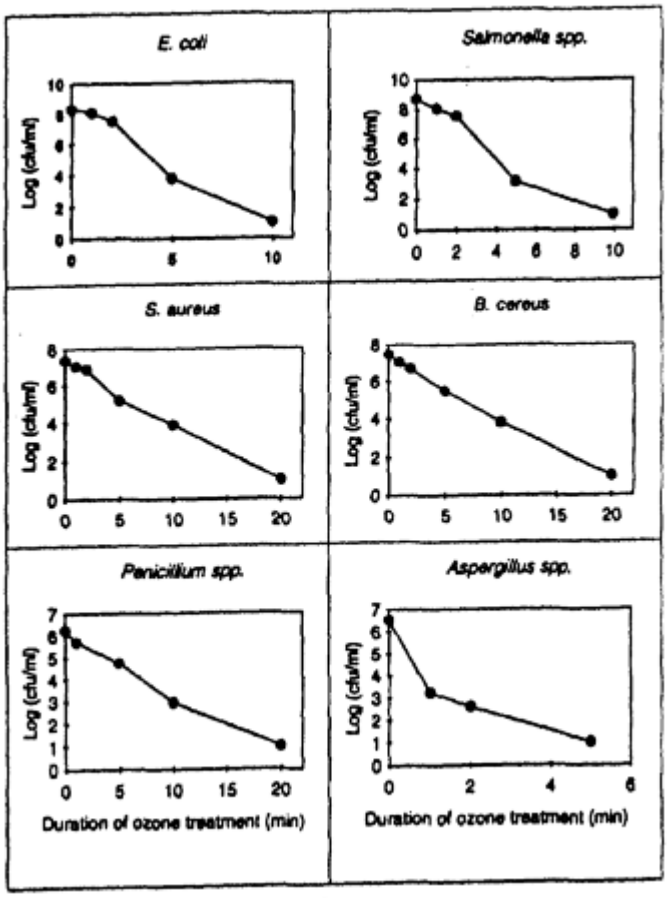


Figure 2.6.08. Kinetics of microbial population reduction in peptone water (1.0 g kg^{-1}) as a result of ozone treatment. Ozone-containing air with an ozone concentration of 6.7 mg $liter^{-1}$ was sparged into the microbial suspensions at an air flow rate of 6 liters min^{-1} (Zhao and Cranston, 1995).

Discussion: The results of this study demonstrate that ozone is effective in reducing levels of microbial populations in black pepper. The effectiveness for ground black pepper depends on the moisture level of the spice. Products having low moisture levels required longer treatment (higher dosage of ozone), but longer treatment resulted in the oxidation of volatile oils of the spice, which would not be acceptable to the spice industry. Ozone, if applied via a change to the traditional spice processing, namely by washing whole peppercorns with ozonized water, does appear to be a viable alternative for decontamination of pepper without affecting the flavor components of the spice.

From a production engineering point of view, whole black peppercorns, often heavily contaminated during harvesting and drying, can be ozone-treated to reduce the initial microbial load. Hygienic processing in later stages would ensure a microbiologically sound product. This method of decontamination also should be applicable to similar spice products.

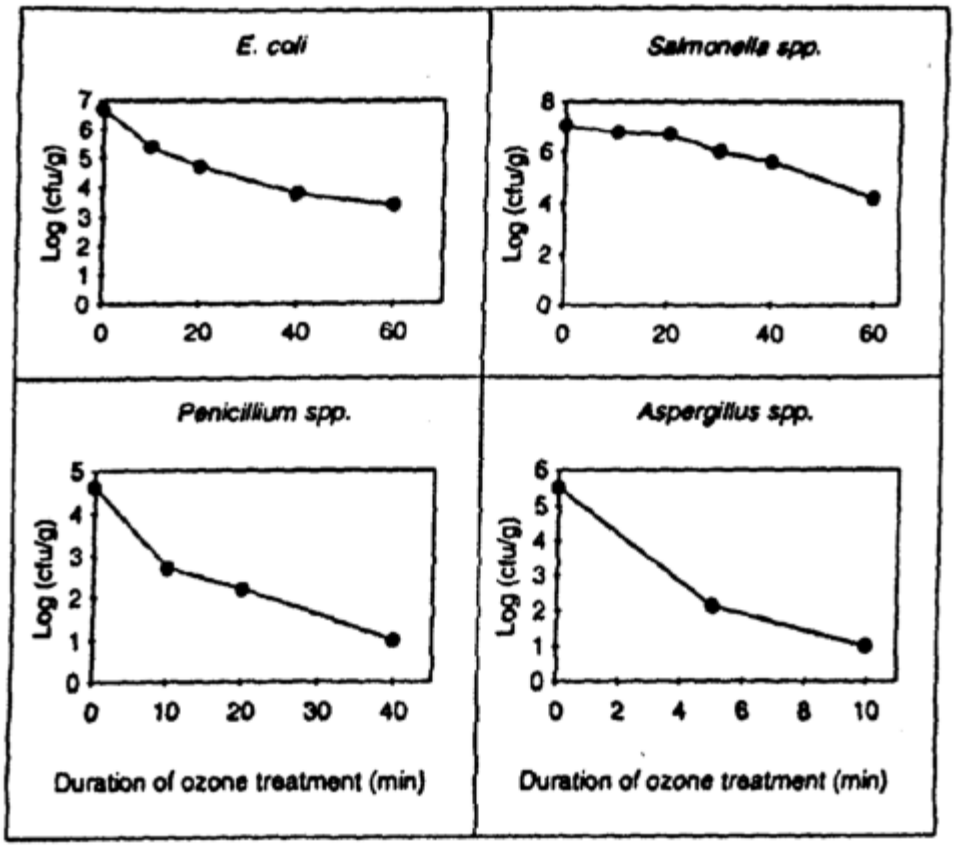


Figure 2.6.09. Kinetics of microbial population reduction in ground black pepper as a result of ozone treatment. The moisture content of the spice was 104.0 g kg^{-1}. Ozone-containing air with an ozone concentration of 6.7 mg $liter^{-1}$ was sparged into the spice at an air flow rate of 6 liters min^{-1} (Zhao and Cranston. 1995).

2.6.2.2 Naito et al. (1987a) – Whole Cloves

This article describes microbiocidal effects of ozone on cereal grains, cereal grain powders (flours), peas, beans and spices. The article is discussed in detail in Section 2.6.2.1. Several of the figures in this article contain microbiocidal data on all of these foods, and the reader of this section is referred to Section 2.6.2.1 for those data related to whole spices. In addition, however, Figure 2.6.12 shows the effect of ozone treatment temperature (5-55EC) on the pasteurization efficiency of whole cloves.

Table 2.6.06. Influence of moisture content of ground black pepper on bactericidal efficiency of ozone (Zhao and Cranston, 1995).

Moisture content of spice (g kg^{-1})	Reduction in microbial population of the spice (log cfu g^{-1})[a]		
	Total aerobic bacteria	Total anaerobic bacteria	Aerobic spore formers
390	383	364	222
1040	511	587	387
1760	619	607	406

[a] The ground black pepper (500 g) was exposed to treatment with ozone-containing air for 6 h at an air flow rate of 6 liters min^{-1}. The concentration of ozone in the air was 6.7 mg $liter^{-1}$.

2.6.3 Broiler Eggs

2.6.3.1 Rauch (1996)

This is a M.Sci. thesis for which only an abstract and Summary and Conclusions section is available. These are presented here.

Abstract: Bacterial numbers on the surfaces of broiler eggs were found to be inversely related to hatchability. Specifically, as bacterial numbers increased, hatchability decreased. Ozone was used to reduce bacterial numbers with the intent of increasing hatchability. Concentrations of 1 to 4 ppm of ozone, as used in the conditions of this study, did not increase hatchability, even though bacterial numbers were reduced. However, duration of exposure appeared to act differently from ozone concentration.. Specifically, 20 min of exposure caused a numerical increase in hatchability when compared to other times of exposure and non-exposed controls. Possibly ozone selectively altered microbial flora such that gas exchange across the shell was increased. Commercial trials are needed to further assess the potential of ozone as a hatchability enhancer of broiler eggs.

Summary and Conclusions: An ozone chamber was constructed of plexiglass. This chamber held one setting tray or one hatching basket for a Jamesway Model 252 incubator. Broiler hatching eggs were placed in this chamber for ozonation. ozone concentrations between 0 and 4 ppm were provided by the ozonation unit attached to the ozonator chamber. Exposure tines ranged between 0 and 20 min.

Initial experiments showed that the microbial flora on commercial broiler eggs was rather constant throughout the incubation period. Attempts to eliminate all bacteria on eggs did not increase hatchability. In fact, our results suggest that a certain microbial flora may be necessary for optimal hatchability of eggs. Therefore, our studies were designed to investigate the reduction, but not elimination, of microbe levels on the surfaces of eggs as they related to hatchability.

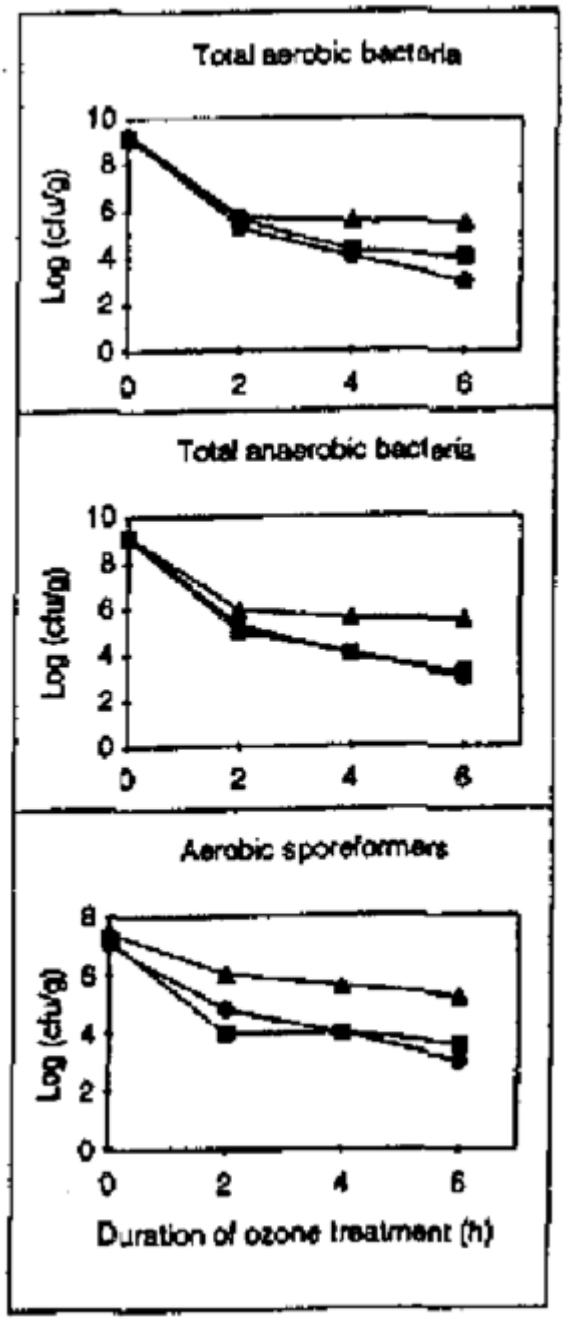


Figure 2.6.10. Influence of moisture content of ground black pepper on the reduction of microbial population in the spice as a result of ozone treatment. ! = 176.0 g kg^{-1} moisture; # = 104.0 g kg^{-1} moisture; = 390 g kg^{-1} moisture (Zhao and Cranston, 1995).

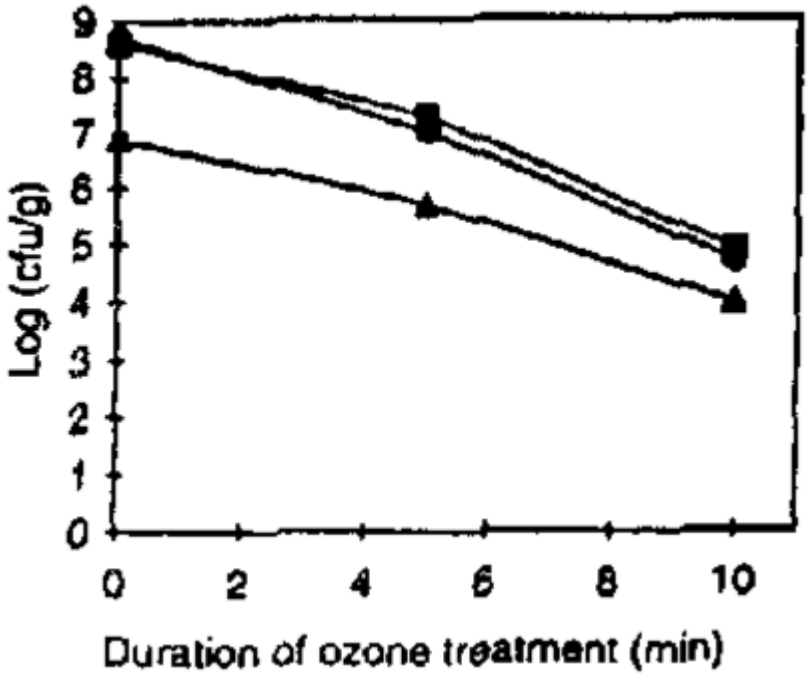


Figure 2.6.11. Reduction of microbial level of whole black peppercorns as a result of steeping the spice in ozonized water. Ozone-containing air with an ozone concentration of 6.7 mg liter^{-1} was sparged into the peppercorn --water mixture at an air flow rate of 6 liters min $^{-1}$. ! = Total aerobic bacteria; # = total anaerobic bacteria; = aerobic spore formers (Zhao and Cranston, 1995).

Initial experimentation showed that the microbial flora on commercial broiler eggs remained rather constant throughout the incubation period in the incubation system used in this project. Reduced bacterial numbers on the surfaces of eggs were associated with increases in hatchability. All concentrations of ozone employed in the experimental system used in this study reduced hatchability as compared to nonozonated control eggs. However, the time required to place eggs in the ozonation chamber and to complete the ozonation process may have resulted in cooling of the eggs. It is possible that this discrepancy accounted for at least a part of the difference in hatchability between ozonated and nonozonated eggs.

It is interesting to note, however, that eggs that were ozonated for 20 minutes, regardless of level of exposure, exhibited an increased level of hatchability. This finding will require additional experimentation, especially under commercial conditions, for verification.

Results of this study must be accepted as a preliminary indicator of the effectiveness of ozonation as a modulator of bacterial populations on hatching eggs. The premise that a preferential flora of bacteria on eggs will increase hatchability and chick quality is based upon the bacteria affecting conditions inherent to the eggshell and eggshell membranes in such a way that exchanges of the essential gases become optimal.

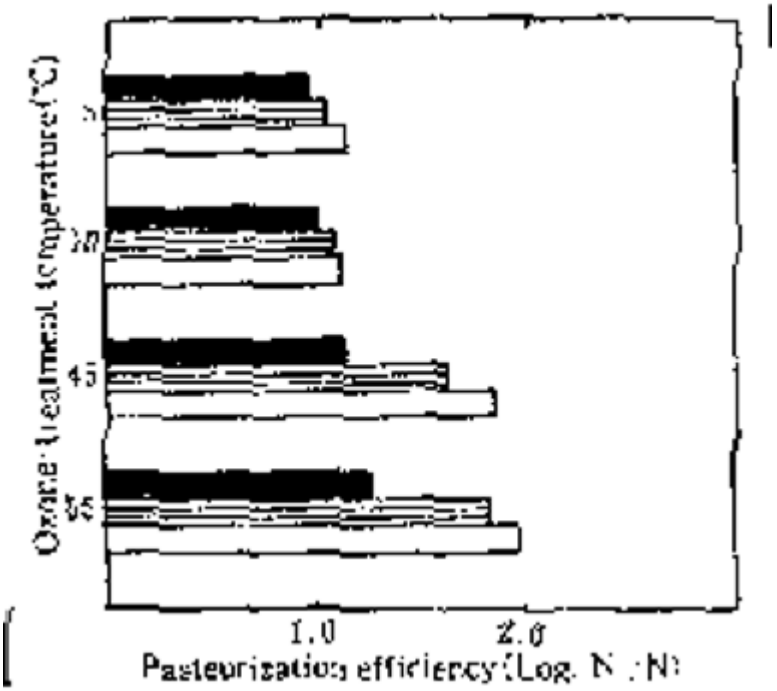


Figure 2.6.12. Effect of ozone treatment temperature on the pasteurization efficiency of whole cloves. Ozone concentration (ppm) : # 0.5; /5; Q 50; ozone treatment time : 1 hour (Naito et al., 1987a).

The results of this study are sufficient to pose a specific intriguing question: "Does bacteriologically-induced physicochemical changes in eggshell membrane permeability to gases and water create an environment that enhances hatchability?" Considerable work employing various levels of ozone with defined numbers and types of microbes will be required. It also may be necessary to monitor the availability of O_2, CO_2, O_3, NH_3, and water vapor to the embryo during incubation.

Ozone remains an enigma as a potential sanitizing agent for hatching eggs. However, results of this study, along with those described by others, indicate that ozone holds potential to enhance the science and art of hatching poultry eggs.

2.6.4 Confectionery Plants – Air Treatment

2.6.4.1 Naitoh (1989d)

Abstract: Ozone treatment was applied to the insides of a confectionery plant at 0.003 ~ 0.112 ppm for 3 years, except during working hours (about 10 per day) to try to establish a convenient method for decontamination. Results were as follows: (1) levels of airborne microorganisms in the cake manufacturing process were reduced by ozone treatment. The predominant strains of airborne microorganisms detected in the cake manufacturing process were *Bacillus, Coliform* bacteria, *Micrococcus* and *Cladosporium*. The microbial contents of the area around the cooking table, packaging table, washing table and cold room were 46, 46, 39, and 41, respectively, per

53-L of air. Especially *Coliform* bacteria, *Micrococcus* and yeast growth was remarkably inhibited by ozone treatment. (2) Levels of airborne microorganisms in the pie manufacturing process were reduced to a lesser extent than that of the cake manufacturing process by ozone treatment. The predominant strains of airborne microorganisms detected in the pie manufacturing process were *Bacillus, Micrococcus* and yeast. The microbial contents in the pie cooling room and oven room were 140 and 55, respectively, per 53-L of air, and after ozone treatment to the insides of the confectionery , the microbial levels were reduced to 78 and 83, respectively, per 53-L of air. *Coliform* bacteria growth was remarkably inhibited by ozone treatment. (3) The predominant strains of airborne fungi were mainly Aspergillus, Penicillium, Cladosporium, and *Rhizopus*. *Cladosporium* was the most predominant strain and accounted to 50~60% of the total fungi. The fungal contents of these four fungi around the mixing machine in July were 2, 2, 6, and 1, respectively, per 53-L of air, and after ozone treatment, the fungal contents were 2, 2, 5, and 2, respectively, per 53-L of air, essentially unchanged.

Experimental Methods

Description of the Locations Where the Airborne Microorganisms Were Measured: A total of 18 rooms on the first and second floors of a cake manufacturing plant located in Aichi Prefecture were selected for airborne microorganism measurement. The airborne microorganisms before ozone treatment were counted in April and July of 1982, and the airborne microorganisms after ozone treatment were counted in April and July of 1986. The shortcake and decoration cake- making room, material keeping room, cold room and refrigeration room were located on the first floor. The pie-making room, material storage room, cold room, refrigeration room, dressing room and lavatory were located on the second floor.

Ozone Treatment: A total of 28 ultraviolet light lamp-type ozone generating systems (a composite tube made of special glass: 1.6 g/hour at 5-10EC ozone generating capacity, fan attached forced convection) were installed on ceilings of the rooms and operated at nights only (5-10 hours/day after working hours) for three years from April 1983 to July 1986. The amount of ozone generation varied, depending upon the temperature, in the range of 1.6 g/hour at 5-10EC, 1.32 g/hour at 10-20EC, and 1.12 g/hour at 20-25EC. The air flow rate changed the ozone concentration; therefore the number of ozone generator installations was increased in places where air flow rate was high, in an attempt to achieve uniformity of ozone concentration throughout the rooms.

Measurement of Ozone Concentration: The ozone concentration was measured using a UV ozone monitor. Ozone decomposes when it comes in contact with walls and equipment and its concentration in the room varies from place to place, depending upon the rate of air flow and the room structure; therefore the measurement of ozone concentration was made in as many places as possible.

Method of Measurement of Airborne Microorganisms: The airborne microorganisms were captured by sucking the air at the rate of 26.5 L/minute for 2 minutes using a pinhole sampler onto plates laid on the table under the samples. Individual plates contained tryptosoy agar medium, potato dextrose agar medium (containing chloramphenicol 30 mg/mL, home-made), and desoxycholate agar medium. Following suction of the air onto the plates containing the agar medium, the plates were incubated: tryptosoy agar plate at 30EC for 3 days, potato dextrose agar

plate at 25EC for 5 days, and desoxycholate agar at 37EC for 1 day. At the end of the incubation, the number of colonies appearing on the plates were counted and recorded as the number of microorganisms.

Identification of Bacteria and True Fungi: The microorganisms grown on the plates which were laid on the table under the pinhole sampler were inoculated and cultured on the plates containing the same kind of agar medium where the organisms were grown, and the process was repeated once more for separation of pure culture. Thus, isolated pure cultures of the organisms were identified to the genus level. True fungi were identified to the genus level through the examination of morphological and biochemical characteristics of the organisms by means of the slide culture method. For identification of intestinal bacteria, the typical peach to red-colored colonies appearing on the plate containing deoxycholate agar medium were inoculated and cultured on the same medium, and the process was repeated once more for pure culture separation. Among the separated organisms, those which were gram-negative asporogenus rods, produced acid and gas in the presence of lactose bouillon and showed oxidase-active negative characteristics, in accordance with the food hygiene law and the description of the *Coli Aerogenes* subcommittee were identified as intestinal bacteria. *Bacillus* and *Micrococcus* were identified in the following manner:. *Catalase* reaction positive rods and spore-forming gram-positive organisms were identified as *Bacillus.* The organisms in which cells were spherical, of uniform size, occurring in singles, in pairs, in tetrads or in small clumps, catalase reaction positive, oxidize glucose and asporogenus gram-positive were identified as *Micrococcus*. The characteristics of spore formation were determined by microscopic examination of the dyed spores.

Experimental Results

Change of Airborne Microorganisms by Ozone Treatment: The results of measurement before the ozone treatment of airborne microorganisms in the plant are presented in Table 2.6.07. The numbers of airborne microbes of *Bacillus*, *Micrococcus*, intestinal bacteria and fungi in the samples collected before the ozone treatment at all of the selected places in July of the warm weather were definitely higher than those of the samples collected in April. Since the finished cake products are not heat-treated, the secondary contamination of cakes by the intestinal bacteria and true fungi is a problem in many cases. It was recognized in the measurement we conducted that the presence of intestinal bacteria and yeast was detectable already in large numbers in the samples collected in April and the numbers increased greatly in the samples collected in the hot and humid July. In the area near the shortcake and decoration-cake-making tables, the presence of a large number of yeast with only a few other microbes was detected in April, but the presence of intestinal bacteria and *Bacillus*, in addition to yeast, in large numbers was detected in July. The airborne microorganism population in the areas next to the cake-making tables, namely the packaging table, washing table and the rack areas was similar to that of the cake-making area. In the refrigeration room and cold room, a large number of *Micrococcus* and yeast were found and they were followed by intestinal bacteria, fungi and *Bacillus* in descending order. In the material keeping room, a large number of *Bacillus* and *Micrococcus* were found and they were followed by yeast and fungi in descending order; very few intestinal bacteria were found.

In the pie-making room on the second floor, large numbers of *Bacillus* were found, followed by *Micrococcus* yeast, fungi and intestinal bacteria in descending order. In the refrigeration room and cold room on the second floor, large numbers of *Bacillus* were found, followed by yeast, *Micrococcus*, fungi and intestinal bacteria in descending order. In the area nearby the ingredients mixing machine and oven, an overwhelmingly large number of *Bacillus* was found. The microbial population in the material keeping room was similar to that of the material keeping room on the first floor, namely consisting primarily of *Bacillus*, *Micrococcus* and yeast. In the lavatory, a large number of intestinal bacteria was found, followed by yeast, *Micrococcus* and *Bacillus* in descending order. In the dressing room, a large number of *Bacillus* was found, followed by yeast and *Micrococcus* in descending order. Immediately after completion of the measurement of microbial population before the ozone treatment, the ozone generating system was installed on the ceiling of each room, generating ozone at night , and the ozone concentration and microbial population were measured in the early mornings just before the ozone generating system was turned off.

The concentration of ozone in each room measured after operating the ozone generating system for three months is presented in Table 2.6.08. Ozone concentrations in the vicinity of the cake-making table, packaging table, washing table and rack area were 0.004 to 0.012 ppm Ozone concentrations in the refrigeration room and cold room of the first floor differed greatly from those of the second floor; those of the first floor were 0.005 ppm and 0.010 ppm, respectively, while those of the second floor were 0.112 ppm and 0.107 ppm, respectively. The differences seemed to be due to the difference in the humidity of the rooms. The ozone concentrations in the material keeping rooms were 0.008 to 0.013 ppm; those in the pie-making room on the second floor were 0.017 ppm; those in the vicinities of the ingredients mixing machine and oven and in the material keeping room were 0.069 to 0.097 ppm. The ozone concentration in the lavatory was 0.047 ppm and that in the dressing room was 0.051 ppm. The ozone concentration of the second floor was comparatively higher than that of the first floor. The reason for this seemed to be due to the low rate of ozone decomposition in the atmosphere of low humidity (50-60% RH), which was maintained because of the pie-making operation on the second floor.

Table 2.6.07. Microbial flora for airborne microorganisms isolated from confectionary (Naitoh, 1989d)

Examination point	**Genus**					**Total micro-organisms**
	Bacillus	***Coliform* bacteria**	***Micrococcus***	**Yeast**	**Mold**	
1	8 (5)	10 (2)	6 (5)	15(10)	4 (4)	46 (28)
	9 (3)	12 (6)	6 (3)	10 (10)	5(4)	48 (30)
3	4 (2)	15 (5)	6 (3)	14 (12)	4 (3)	49 (29)
4	3 (1)	10 (4)	5 (2)	16 (15)	1 (0)	39 (25)
5	3 (1)	13 (3)	5 (2)	12 (10)	4 (2)	40 (20)
6	4 (1)	7 (3)	12 (5)	12 (6)	5 (3)	42 (22)
7	5 (2)	8 (6)	10 (4)	10 (5)	6 (3)	41 (22)
8	25 (12)	2 (0)	25 (11)	21 (18)	12 (10)	89 (55)
9	30 (17)	3 (0)	36 (12)	28 (18)	15 (12)	115 (78)
10	38 (10)	4 (2)	52 (20)	30 (27)	12 (10)	140 (70)
11	51 (21)	2 (1)	28 (20)	22 (20)	10 (8)	124 (75)
12	39 (25)	5 (2)	12 (6)	29 (21)	6 (5)	105 (68)
13	53 (20)	12 (5)	25 (10)	31 (25)	19 (12)	146 (75)
14	48 (12)	10 (2)	10 (5)	29 (20)	12 (10)	115 (55)
15	26 (10)	2 (0)	6 (3)	8 (4)	7 (6)	53 (25)
16	29 (19)	7 (3)	27 (13)	24 (20)	11 (5)	105 (63)
17	27 (20)	59 (12)	35 (18)	46 (26)	9 (7)	182 (93)
18	43 (15)	16 (8)	31 (18)	34 (28)	12 (10)	136 (85)

Figures in the table are microbial counts per 53-L of air. The numbers represent microorganism counts in July. Numbers in parentheses represent microorganism counts in April.

Table 2.6.08. Ozone concentration at the manufacturing process (Naitoh, 1989d)

Examination point	Average ozone concentration (ppm)	
	April	July
1	0.005 ∀ 0.002	0.003 ∀ 0.002
2	0.007 ∀ 0.003	0.005 ∀ 0.003
3	0.005 ∀ 0.002	0.004 ∀ 0.002
4	0.004 ∀ 0.001	0.003 ∀0.002
5	0.12 ∀ 0.005	0.008 ∀ 0.004
6	0.005 ∀ 0.002	0.005 ∀0.002
7	0.010 ∀ 0.03	0.007 ∀ 0.003
8	0.013 ∀ 0.005	0.008 ∀ 0.003
9	0.010 ∀ 0.002	0.010 ∀ 0.005
10	0.008 ∀ 0.002	0.005 ∀ 0.003
11	0.017 ∀ 0.006	0.015 ∀ 0.007
12	0.112 ∀ 0.025	0.094 ∀ 0.012
13	0.107 ∀ 0.021	0.072 ∀ 0.021
14	0.097 ∀ 0.012	0.086 ∀ 0.020
15	0.086 ∀ 0.021	0.049 ∀ 0.010
16	0.069 ∀ 0.012	0.047 ∀ 0.015
17	0.047 ∀ 0.010	0.085 ∀ 0.022
18	0.051 ∀ 0.022	0.063 ∀ 0.017
Ozone was determined by means of a UV monitor.		

From the results of measurement of airborne microorganisms, a general trend toward reduction of intestinal bacteria, yeast and *Micrococcus* by the ozone treatment was recognized. The results of airborne microorganisms measured approximately three years after ozone treatment was started are presented in Table 2.6.09. The intestinal bacteria and yeast, which were the predominant organisms before ozone treatment, were greatly decreased after ozone treatment. This trend appeared noticeably at the cooking table, packaging table, washing table and in the vicinity of the rack, intestinal bacteria were not detected in April measurements; yeast population was decreased to 1/2-1/3 of the initial count; and *Bacillus* and *Micrococcus* also were decreased. However, very little change in fungi count was recognized. In the refrigeration room and cold room on the first floor, intestinal bacteria were not detected in both the April and July measurements and yeast was decreased to about ½ of the initial count. In the material keeping room on the first floor, *Bacillus* and *Micrococcus*, which were the predominant organisms before ozone treatment, were decreased to 1/2-1/3 of the initial count. In the pie-making room on the second floor, *Bacillus* and *Micrococcus* were decreased, and in the refrigeration room and cold room on the same floor, no intestinal bacteria was detected. In the ingredients mixing table on the second floor, *Bacillus* and yeast, which were the predominant organisms before ozone treatment, were decreased to about 1/2 of the initial count; *Bacillus* in the vicinity of oven was

decreased, and in the material keeping place *Bacillus*, *Micrococcus* and yeast were decreased to about 1/2 of the initial counts. In the lavatory on the second floor, intestinal bacteria were greatly decreased; and in the dressing room, all of the test microorganisms, with an exception of fungi, were decreased to about 1/2 of the initial counts.

Change of Airborne Fungi by Ozone Treatment: Airborne fungal contents which were comparatively difficult to sterilize with ozone treatment were measured. The results of measurement of the fungal contents at six places where a relatively large number of fungi were counted after the ozone treatment, namely, material keeping place, pie- making room, cold room, ingredients mixing table, material keeping place and dressing room, are presented in Table 2.6.10. Four strains of fungi detected at the six places were *Aspergillus, Penicillium, Cladosporium* and *Rhizopus.* The change by ozone treatment of fungi count and fungal contents in material keeping place, ingredients mixing table and pie-making room was relatively minute. However, in the cold room, *Cladosporium* was the predominant fungi before the ozone treatment ranging from 63.2 to 66.7% of the fungal contents, but it decreased after the ozone treatment to 50.0-53.3% of the fungal contents. *Cladosporium* which was predominant in the dressing room also decreased after ozone treatment.

Discussion

Airborne microorganisms originate mainly in soil and raw materials of foods and also are carried by human beings and animals. Airborne microorganisms comprise organisms resistant especially to the sterilizing effects of ultraviolet rays and desiccation and capable of surviving such an adverse environment. Therefore it is expected that bacterial spores, yeast spores and fungi make up a large portion of airborne microorganisms. In food processing plants where breads, cakes and semi-bakes are manufactured, the bacterial, yeast and fungal contamination of the products is a serious problem. The main airborne microorganisms in the cake manufacturing plant where the author conducted his present investigation were *Bacillus*, *Micrococcus,* intestinal bacteria, yeast and fungi. A large number of intestinal bacteria and yeast were found in the areas of the cake-making table, packaging table, washing table, in the vicinity of the rack on the first floor where a large amount of water was consumed; and the microbial numbers measured in July were 10-5 per53-L and 10-16 per 53-L, respectively. However, after the ozone treatment, intestinal bacteria were not detected and yeasts were greatly reduced to about 1/2-1/3 of the initial count. This was thought to be due to the low resistance level of these organisms to ozone and the enhanced sterilizing effect of ozone resulting from a rapid decomposition of ozone in the atmosphere of high humidity (70-80%) in July.

Table 2.6.09. Effect of ozone treatment of microbial flora for airborne microorganisms isolated from a confectionary plant (Naitoh, 1989d)

Examination point	**Genus**					**Total micro-organisms**
	Bacillus	***Coliform* bacteria**	***Micrococcus***	**Yeast**	**Mold**	
1	2 (1)	0 (0)	3 (2)	5 (2)	5 (3)	18 (10)
2	3 (2)	2 (0)	2 (0)	4 (1)	5 (5)	19 (11)
3	2 (0)	0 (0)	2 (1)	7 (4)	2 (1)	16 (8)
4	1 (0)	0 (0)	3 (2)	3 (2)	7 (2)	16 (10)
5	3 (0)	0 (0)	2 (0)	5 (3)	6 (3)	18 (8)
6	2 (1)	0 (0)	6 (2)	2 (1)	7 (3)	19 (9)
7	3 (0)	0 (0)	5 (3)	5 (3)	6 (4)	22 (12)
8	15 (8)	0 (0)	7 (3)	10 (8)	13 (12)	48 (35)
9	10 (6)	0 (0)	9 (2)	12 (8)	14 (12)	47 (30)
10	12 (7)	0 (0)	18 (5)	13 (9)	15 (13)	38 (30)
11	30 (15)	0 (0)	15 (12)	18 (12)	10 (8)	62 (28)
12	22 (12)	0 (0)	10 (3)	15 (12)	5 (6)	78 (50)
13	32 (18)	2 (0)	11 (4)	16 (10)	15 (4)	55 (38)
14	20 (5)	2 (0)	3 (2)	15 (10)	13 (12)	79 (40)
15	17 (3)	0 (0)	1 (0)	3 (2)	8 (6)	56 (32)
16	13 (9)	0 (0)	10 (3)	12 (8)	10 (2)	33 (15)
17	17 (9)	15 (3)	12 (9)	10 (8)	5 (3)	46 (25)
18	20 (12)	3 (0)	12 (8)	12 (7)	10 (7)	65 (35)

The figures of the table are microbial counts per 53-L of air. The numbers represent counts of microorganisms in July. The numbers in parentheses represent counts of microorganisms in April. Ozone treatment period : 3 years (but only during nighttime). Ozone treatment concentration : 0.003 ~ 0.112 ppm.

Table 2.6.10. Effect of ozone treatment on distribution of airborne fungi in the confectionary plant (Naitoh, 1989d)

Examination point	Control					Ozone				
	A	P	C	R	O	A	P	C	R	O
8 April	1 (10.0)	1 (10.0)	5 (50.0)	2 (20.0)	1 (10.0)	2 (16.7)	2 (16.7)	5 (41.7)	2 (16.7)	1 (8.3)
July	1 (8.3)	2 (16.7)	6 (50.0)	1 (8.4)	2 (16.7)					
11 April	1 (12.5)	2 (25.0)	4 (50.0)	0 (0.0)	1 (12.5)	1 (12.5)	2 (25.0)	4 (37.5)	1 (12.5)	2 (12.5)
July	1 (10.0)	2 (20.0)	5 (50.0)	1 (10.0)	1 (10.0)	1 (10.0)	2 (20.0)	4 940.0)	1 (10.0)	2 (20.0)
13 April	1 (8.3)	2 (16.7)	8 (66.7)	0 (0.0)	1 (8.3)	0 (0.0)	1 (25.0)	2 (50.0)	1 (25.0)	0 (0.0)
July	1 (5.3)	3 (15.8)	12 (63.3)	1 (5.3)	2 (10.2)	2 (13.3)	2 (20.0)	8 (53.3)	1 (6.7)	1 (6.7)
14 April	2 (20.0	1 (10.0)	3 (30.0)	2 (20.0)	2 (20.0)	2 (13.3)	5 (33.3)	5 (33.3)	2 (13.3)	1 (6.7)
July	2 (16.7))	2 (16.7)	6 (50.0)	1 (8.3)	1 (8.3)	2 (15.4)	2 (15.4)	5 (61.5)	2 (15.4)	2 (15.4)
16 April	1 (20.0)	0 (0.0)	2 (40.0)	1 (20.0)	1 (20.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)
July	2 (18.2)	1 (9.0)	5 (45.5)	1 (9.0)	2 (18.2)	1 (10.0)	2 (20.0)	4 (40.0)	1 (10.0)	2 (20.0)
18 April	2 (20.0)	1 (10.0)	5 (50.0)	1 (10.0)	1 (10.0)	1 (14.3)	2 (28.6)	2 (28.6)	1 (14.3)	1 (14.3)
July	2 (16.7)	2 (16.7)	3 (25.0)	2 (16.7)	3 (25.0)	1 (14.3)	2 (28.6)	3 (42.9)	1 (14.3)	0 (0.0)

The figures of the table are microbial counts per 53-L of air. Numbers in parentheses are % of each parameter to total isolation. A = *Aspergillus*; P = *Penicillium*; C = *Clostridium*; R = *Rhizopus*; O = Others. Control : before ozone treatment; Ozone : ozone treatment.

A large number of *Micrococcus* and yeast was found in the refrigeration room and cold room on the first floor and the microbial number of organisms including bacteria and true fungi measured in July were 42-43 per 53-L. However, after ozone treatment, no intestinal bacteria were found, *Micrococcus* and yeast counts were down to one-half of the initial counts and the microbial counts including bacteria and true fungi were also down to 19-22 per 53-L, which was ½ of the initial counts. This was thought to be due to the low resistance level of the predominant microorganisms, *Micrococcus*, yeast, and intestinal bacteria, to ozone and the enhancement of

the sterilizing effect of ozone resulted from the rapid decomposition of ozone occurring in the high humidity atmosphere (concentration of the remaining ozone: 0.005-0.01 ppm), although ozone does not decompose readily in low humidity atmosphere). In the refrigeration room and cold room, large numbers of *Bacillus, Micrococcus* and yeast were found and the total microbial counts in July were 91-115 per 53-L, but that number was decreased to 55-78/53 L after the ozone treatment.

A large number of *Micrococcus* and yeast, the airborne microorganisms thought to be carried by raw cream, were found in the refrigeration room and cold room on the first floor, since mainly raw cream entered the rooms. The resistance level of these organisms to ozone is low and the humidity of the rooms was high (60-80% RH), therefore it was thought that ozone in the rooms decomposed rapidly and enhanced its sterilizing effect. On the other hand, a large number of *Bacillus, Micrococcus* and yeast, the airborne microorganisms thought to be carried by the raw materials for pie manufacturing, such as wheat flour, were found in the refrigeration room and cold room on the second floor, since mainly wheat flour and other raw materials for pie making entered these rooms. The humidity in these rooms was low (55-60% RH), which restrained ozone decomposition, leaving the remaining ozone concentration high in the range of 0.072-0.112 ppm; it was thought that the sterilizing effect of ozone in these rooms dropped somewhat in comparison with that of the refrigeration room and cold room on the first floor. Distribution of ozone in a room is greatly influenced by the room structure and flow of air, since ozone is decomposed through its contact with water molecules and also decomposed physically through its contact with the walls and equipment. For example, the varying levels of ozone concentration found at different parts of the refrigeration room and cold room on the second floor may be explained as follows:

The highest ozone concentration was found near the floor of the rooms. This occurred because ozone's molecular weight is 48 and it is heavier than the air The second highest ozone concentration was found near the walls of the rooms. It was thought that, although ozone decomposes through its contact with the walls, its decomposition was so slow due to the low temperature that the undecomposed ozone adhered to the wall. When water accumulated on the walls, the amount of ozone adhering to the walls is reduced and the ozone concentration near the walls drops to a level lower than that of the ozone concentration in the middle of the rooms [the refrigeration room and cold room on the 1st floor].

The resistance level of fungi is considered to be relatively high, although the level varies depending upon the strains. It was thought that, although the sterilizing power of ozone varies depending upon the condition of treatment, the resistance level to ozone of *Cladosporium* is the highest, which is followed by *Aureobasidium, Alternaria, Geotrichum, Trichoderma, Wallemia, Aspergillus* and *Penicillium* in descending order. The main strains of fungi detected in the plant where the author's investigation was conducted were the four strains - *Aspergillus, Penicillium, Cladosporium*, and *Rhizopus*. *Cladosporium* was the predominant organism both before and after the ozone treatment. The *Cladosporium* count was lowered in some places in some degree by the ozone treatment, but it seemed to be due to presence of *Cladosporium* in larger numbers than the other strains of fungi. Although the fungal counts varied somewhat depending upon the place and time of measurement, three years of ozone treatment at the low level of ozone concentration (0.01-0.1 ppm) limited to nights only in the plant where the fungal counts were

relatively low did not bring any great change in fungal population, although there seemed to be a trend toward decreasing the fungal count to some degree.

Summary

The ozone generating system of the ultraviolet lamp-type was installed in a cake manufacturing plant and inside the plant was treated with ozone at the level of 0.003-0.112 ppm ozone concentration at night (approximately 10 hours/day after working hours) for three years and the change of airborne microorganisms was measured with the following results:

1. In the cake-making area on the first floor where a large amount of water was used, ozone decomposed effectively in the high humidity, enhancing its sterilizing effect, and the ozone treatment greatly reduced the microbial counts, especially of the intestinal bacteria, *Micrococcus* and yeast.

2. In the pie-making room on the second floor, large numbers of *Bacillus, Micrococcus* and yeast, the organisms carried by the raw materials for pie making, were detected. Although these microbial counts were lowered by the ozone treatment, the humidity of the rooms was relatively low, resulting in slower ozone decomposition and its sterilizing effect was lowered somewhat compared with that of the first floor where the humidity was high.

3. In the cake manufacturing plant, the fungal count was relatively low and the main fungi detected were four strains, namely *Aspergillus, Penicillium, Cladosporium* and *Rhizopus*; *Cladosporium* was the predominant strain both before and after the ozone treatment, and a slight trend toward reduction of *Cladosporium* levels by the ozone treatment was noticed. Although the fungal counts and fungal contents varied somewhat depending upon the place and time of measurement, because of the low level of fungal population in the plant, any great reduction of fungi levels or any change in the fungal contents by the treatment with ozone at the low level of concentration was not recognized in this investigation.

2.6.5 Rice Cakes

2.6.5.1 Naitoh (1987)

Recently, deoxygenating substances are used for rice cake packaging. But these type of products are transferred in anaerobic condition, therefore, there is no microbial problem by mold. However, as the storage time increased, microbial spoilage and change of food was prominent, thus ozone treatment was tested for this product.

Rice cakes were treated with ozone at 1, 25, and 50 ppm right after production and were packaged with deoxygenating substances and stored at 5EC. Change of anaerobic and aerobic microorganisms during storage time is shown in Figures. 2.6.13 and 2.6.14. Growth of aerobic microorganisms was inhibited during the storage when more than 25 ppm ozone was applied but in case of anaerobic microorganisms, their growth was inhibited more as the applied ozone concentration was higher. After treating with ozone at 1, 25, and 50 ppm for 6 hours at 5EC, sticky rice was used for making rice cakes. Change of aerobic and anaerobic microorganisms

was investigated (Figures. 2.6.15 and 2.6.16). Similar results were obtained from ozone-treated products.

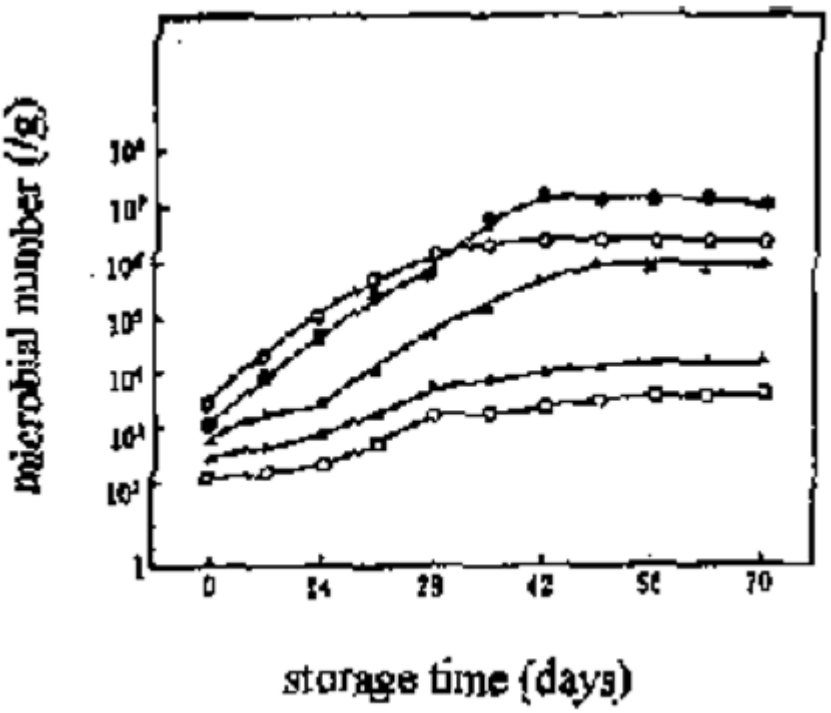


Figure 2.6.13. Change of aerobic microorganisms in ozone-treated rice cake. " control; ! air-30 min;) ozone 1 ppm, 30 min; ozone 25 ppm, 30 min; Q ozone 50 ppm, 30 min (Naitoh, 1987).

2.6.6 Cheeses

2.6.6.1 Gibson et al. (1960)

Controlled studies were carried out at the Dairy Technology Research Institute, Canada Dept. of Agriculture, Ottawa, on the effects of ozone for controlling molds on cheddar cheeses.

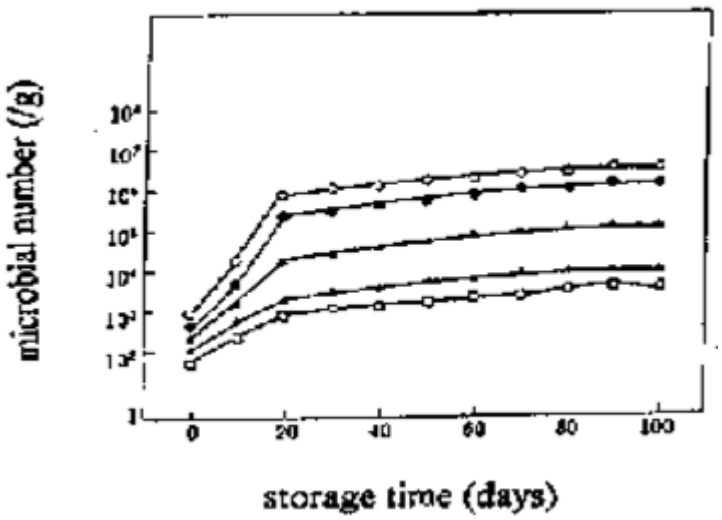


Figure 2.6.14. Change of anaerobic microorganisms in ozone-treated rice cakes. " control; ! air-30 min;) ozone 1 ppm, 30 min; ozone 25 ppm, 30 min; Q ozone 50 ppm, 30 min (Naitoh, 1987).

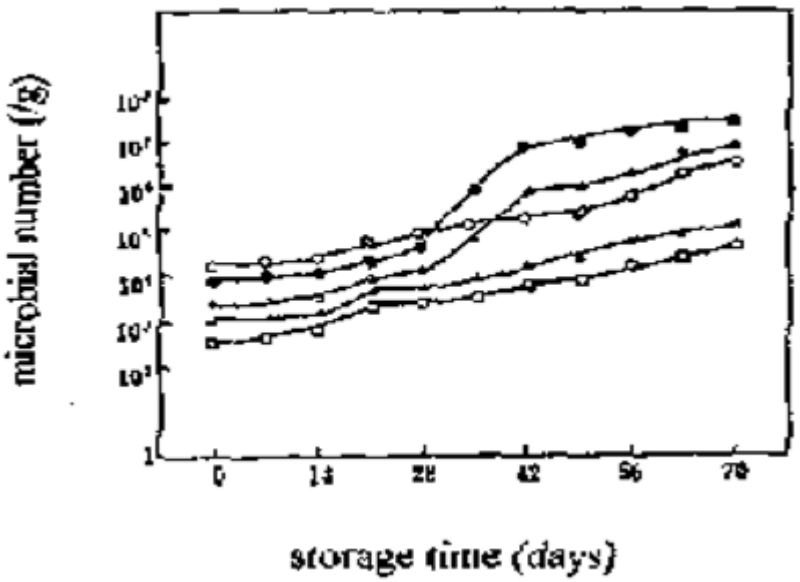


Figure 2.6.15. Change of aerobic microorganisms in cake made with ozone-treated sticky rice. " control; ! air-30 min;) ozone 1 ppm, 30 min; ozone 25 ppm, 30 min; Q ozone 50 ppm, 30 min (Naitoh, 1987).

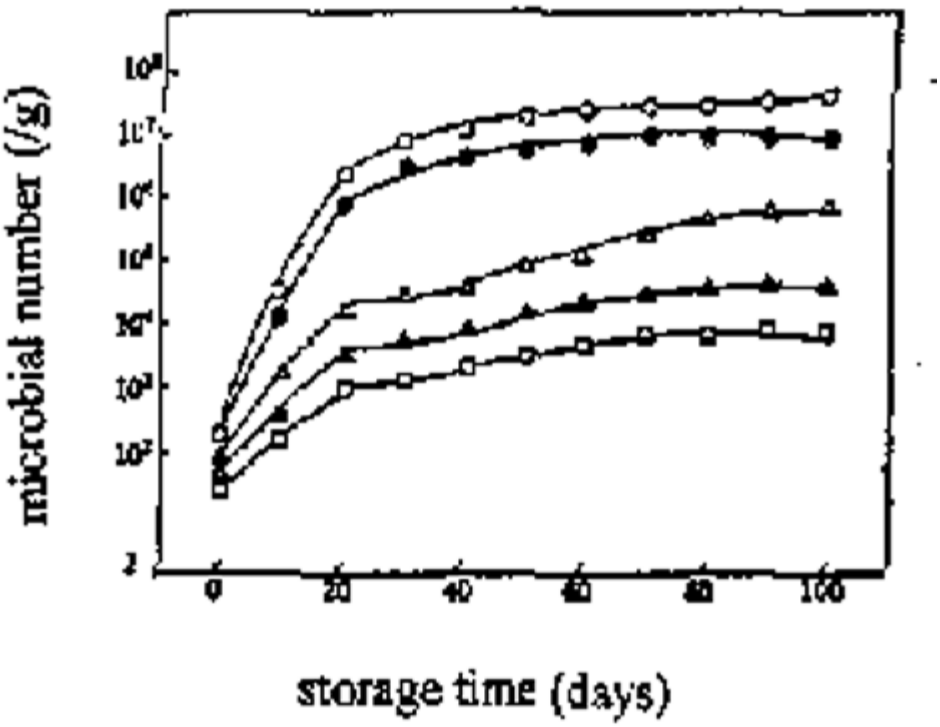


Figure 2.6.16. Change of anaerobic microorganisms in cake made with ozone-treated sticky rice. " control; ! air-30 min;) ozone 1 ppm, 30 min; ozone 25 ppm, 30 min; Q ozone 50 ppm, 30 min (Naitoh, 1987).

Experimental Procedure

Two levels of ozone concentration were employed. An ozone concentration of between 3 and 10 ppm. was used to combat a heavy established mold growth, while one of 0.2-0.3 ppm was used to inhibit mold development. Each experiment was repeated using alternate rooms for treatment and control.

High (3-10 ppm) Level of Ozone: Sixteen Stilton-size cheese were assigned at random into two groups of eight: four waxed and four unwaxed cheese were placed in each of two curing rooms. One room served as a control, the other room was treated with a high concentration (3-10 ppm) of ozone.

Before the treatment began, the rooms and cheese were inoculated with mold spores, which were allowed to germinate and grow until well established. The mold spores used to contaminate the rooms and cheese were collected by scraping the ends of commercial cheeses, which had become molded in improperly managed curing rooms. To inoculate the rooms the molds were first dried at room temperature, then distributed throughout the rooms with the aid of an electric fan.

In order to assess the effectiveness of each treatment, the percentage of the total area of each cheese, which was covered with mold, was estimated. Free mold spore counts in the air in each

room were carried out twice a week in addition to those made at the beginning and end of the experiment. Malt agar plates acidified to pH 3.5, exposed for one minute on the top and bottom shelves in the rooms and incubated at room temperature for 72 hours were used to compare the mold spore concentration in the air of the two rooms.

Throughout the experiment an average room temperature of 61EF and a relative humidity of 83 percent were maintained in the ozone-treated rooms; in the untreated rooms the average temperature and relative humidity were 59.5EF and 87 percent, respectively. Ozone determinations were made two or three times weekly.

To ascertain the effect of ozone on the flavor of the cheese, the cheese used in the first part of the 3-10 ppm experiment were carried through the second replication of the same experiment. At the end of that time they were examined by the federal dairy produce graders.

Low (0.2-0.3 ppm) Level of Ozone: Sixteen Stilton-size cheeses were placed in each of two curing rooms. Eight cheeses were waxed and eight unwaxed, four of each uninoculated and four inoculated with mold spores by placing them in a mold-contaminated curing room for six days before beginning the trial. In addition, 40 one-pound cut pieces of cheese, twenty waxed and twenty wrapped in Saran film were divided between the rooms. Half of each group were inoculated with mold spores by exposing them to the air for 10 minutes in a mold-contaminated curing room and half were uninoculated. The same rooms, materials and procedures were used as in the trials with a high level of ozone.

Results and Discussion

High (3-10 ppm) Ozone Concentration: The percentage area molded on treated and untreated, waxed and unwaxed, ten-pound cheddar cheeses in each test is shown in Table 2.6.11. The values are the averages of the four cheeses in each treatment lot for each test. Only a slight growth of mold developed on the waxed cheese while the unwaxed cheese became heavily molded prior to the ozone treatment. The effect of ozone on mold growth was most evident on the sides of the unwaxed cheese. The cheese underwent a striking change in appearance during the treatment. The mold became bleached and eventually appeared as a white, powdery or colorless, greasy film on the cheese. The ends of the cheese developed a white to light-brown scaly appearance that did not disappear within the treatment times used. Pigmentation was evident on the bottom of the cheese where the shelf afforded some protection from the ozone. A few days after the ozone treatment was stopped, a profuse growth of mold developed on the cheese, indicating that the molds were not destroyed.

Both the waxed and unwaxed cheese became molded in the absence of ozone. The waxed cheese developed a thin surface film of mold which was not injurious to quality. The mold was readily removed from the wax by wiping. The unwaxed cheese incurred serious mold contamination.

Table 2.6.11. The effect of ozone (3-10 ppm) on the molded area of waxed and unwaxed 10-lb cheddar cheese (Gibson et al., 1960)

Trial No.	Days of Treat-ment	Untreated Cheese (no Ozone)				Treated Cheese (3-10 ppm)			
		Waxed*		Unwaxed*		Waxed*		Unwaxed*	
		Ends	Sides	Ends	Sides	Ends	Sides	Ends	Sides
Average Percentage of Molded Area									
I	0	1.5	0.2	96.2	58.7	1.9	0.5	96.2	80.0
	7	1.6	0.06	97.2	92.5	0.3	0.5	93.7	58.7
	14	1.3	0.8	100.0	100.0	0.0	0.0	46.2	0.2
	21	18.7	10.0	100.0	100.0	0.3	0.0	82.8	2.0
	25	34.9	5.2	100.0	100.0	0.8	0.0	84.8	1.2
II	0	3.0	7.0	100.0	100.0	1.1	1.2	100.0	100.0
	9	79.3	38.6	100.0	100.0	0.0	0.0	100.0	99.5
	16	78.7	54.7	100.0	100.0	0.0	0.0	64.1	57.5
	30	74.3	37.5	100.0	100.0	0.0	0.0	20.0	0.0

* Average of four cheeses.

Low (0.2-0.3 ppm) Ozone Concentration: Table 2.6.12 shows the area molded on treated and untreated waxed and unwaxed ten-pound cheddar cheeses in four trials. The values given are the averages for the four cheeses in each treatment lot for each trial. It will be observed that the mold growth was hindered from developing on the sides of the unwaxed cheeses. The ends of the cheeses received only slight protection. Mold was prevented from developing on the sides of the treated waxed cheese and was greatly restricted on the ends in trials I, II and IV; in trial III the treatment was much less effective on the ends. The mold which did develop on the waxed cheese in both treatments took the form of a fine film which did not penetrate the wax and could be removed very readily from the waxed cheese surface. The unwaxed cheese in the control treatment became badly molded.

The percentage area molded on waxed versus film-wrapped, mold-inoculated and uninoculated, ozone-treated and untreated one-pound cut pieces of cheddar cheese is shown in Table 2.6.13. The values are averages of five cheeses in each treatment lot for each trial. In these trials the ozone inhibited the mold growth on mold inoculated and uninoculated waxed cheese. These results are in accordance with those presented in Table 2.6.12. Mold growth on the inoculated and uninoculated film wrapped cheese was almost completely prevented with little difference between the controls and the treated pieces.

Free mold spores were reduced in numbers in the ozone-treated rooms. When 3-10 ppm of ozone was used to combat existing heavy mold growth, the average was 167 colonies per plate in the untreated room and 10 colonies per plate in the treated room. When 0.2-0.3 ppm of ozone was used to inhibit mold growth the average mold spore count was 65 colonies per plate in the untreated room and 8 colonies per plate in the treated room.

Table 2.6.12. The effect of ozone (0.2-0.3 ppm) on the molded area of waxed and unwaxed ten-lb cheddar cheese (Gibson et al., 1960).

Trial No.	Days of Treatment	Untreated Cheese (no Ozone)				Treated Cheese (0.2-0.3 ppm)			
		Waxed*		Unwaxed*		Waxed*		Unwaxed*	
		Ends	Sides	Ends	Sides	Ends	Sides	Ends	Sides
	Average Percentage of Molded Area								
I	0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
	10	0.3	0.0	50.0	36.2	0.2	0.0	10.6	0.0
	18	0.3	0.1	99.7	100.0	0.5	0.0	59.7	7.2
	24	0.7	0.1	100.0	100.0	0.4	0.0	66.2	7.5
	28	1.0	0.2	100.0	100.0	0.1	0.0	70.0	4.0
	35	0.3	0.1	100.0	100.0	0.3	0.0	73.1	7.7
	45	0.2	0.1	100.0	100.0	0.1	0.0	77.6	27.7
	52	0.2	0.1	100.0	100.0	0.5	0.0	89.7	35.0
	59	0.4	0.2	100.0	100.0	0.2	0.0	86.7	37.1
II	0	0.0	0.0	52.5	6.8	0.0	0.0	30.0	0.2
	7	0.2	0.0	99.6	47.0	2.0	0.0	56.2	13.0
	14	3.7	0.0	100.0	100.0	1.8	0.0	63.1	17.5
	24	6.2	0.0	100.0	100.0	3.5	0.0	86.3	49.2
	28	8.7	0.0	100.0	100.0	4.4	0.0	85.7	48.0
	35	57.5	1.7	100.0	100.0	12.2	0.5	91.8	57.7
	42	63.7	11.2	100.0	100.0	19.3	1.0	91.6	52.5
	49	84.3	16.2	100.0	100.0	18.8	0.3	97.1	59.5
	56	79.3	16.2	100.0	100.0	18.7	0.2	96.2	57.0
	63	79.5	36.2	100.0	100.0	39.0	0.2	95.1	60.0
III	0	43.7	0.0	69.3	0.1	61.2	0.0	79.3	0.0
	10	36.8	0.0	95.5	47.5	70.0	0.0	79.5	0.0
	18	38.1	0.1	100.0	98.5	75.6	0.0	89.3	1.7
	24	44.3	0.1	100.0	100.0	79.6	0.0	93.0	6.0
	28	36.2	0.0	100.0	100.0	70.6	0.0	89.7	8.5
	35	52.5	0.0	100.0	100.0	73.7	0.0	92.6	17.7
	45	43.1	0.1	100.0	100.0	75.6	0.0	94.0	35.0
	52	46.8	0.1	100.0	100.0	76.0	0.0	92.0	41.5
	59	46.2	0.2	100.0	100.0	76.6	0.0	94.5	41.2
IV	0	17.3	0.0	96.2	100.0	13.1	0.0	43.7	0.0
	7	16.5	0.0	100.0	100.0	5.4	0.2	81.2	18.7
	14	29.2	0.4	100.0	100.0	15.9	0.1	84.7	14.2
	24	31.6	0.4	100.0	100.0	19.6	0.1	91.3	46.2
	28	30.7	0.5	100.0	100.0	19.1	0.1	87.5	45.2
	35	65.0	1.0	100.0	100.0	20.2	0.5	96.2	54.0
	42	63.1	12.5	100.0	100.0	19.2	2.7	92.5	48.8
	50	85.6	7.7	100.0	100.0	20.6	0.2	97.1	52.5
	56	88.1	11.7	100.0	100.0	21.7	0.2	93.1	52.5
	63	87.5	25.0	100.0	100.0	19.8	0.0	95.8	51.2

* Average of four cheeses.

No flavor defect attributable to the ozone could be detected in the cheese when examined by the federal dairy produce graders.

Table 2.6.13. The effect of ozone (0.2-0.3 ppm) on waxed vs film wrapped, mold inoculated and uninoculated 1-lb pieces of cheddar cheese (Gibson et al., 1960)

Trial No.	Days of Treat-ment	Inoculated				Uninoculated			
		Treated (0.2-0.3 ppm)		Untreated		Treated (0.2-0.3 ppm)		Untreated	
		Waxed*	Film*	Waxed*	Film*	Waxed*	Film*	Waxed*	Film*
		Average Percentage of Molded Area							
I	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	10	0.0	0.0	0.1	1.0	0.0	0.3	0.0	0.1
	18	0.5	0.2	10.2	2.4	0.5	1.2	10.0	1.2
	24	0.7	0.7	18.7	1,8	1.0	1.2	16.2	1.5
	28	1.2	0.5	19.5	1.3	1.7	0.7	22.5	1.5
	35	1.2	0.3	26.2	1.8	1.6	0.8	26.2	2.2
	45	2.1	0.5	25.0	2.7	4.5	2.1	27.5	1,7
	52	5.7	0.3	23.7	2.7	8.0	1.7	28.7	2.7
	59	3.6	0.5	30.0	2.1	8.7	1.2	37.5	1.6
II	0	0.0	0.5	0.0	0.5	0.0	0.1	0.0	0.0
	7	1.2	1.2	5.3	4.7	1.1	0.1	1.3	0.1
	14	2.5	1.6	13.5	3.8	1.1	1.0	9.2	1.7
	24	2.5	1.8	21.5	4.5	2.6	1.2	6.7	1.8
	28	3.3	1.7	25.0	4.7	2.0	1.3	13.2	1.5
	35	5.1	1.6	40.0	6.2	6.5	2.6	36.2	1.0
	42	4.1	1.2	40.0	5.2	5.2	2.2	35.0	1.0
	50	6.7	1.1	38.7	4.1	6.5	1.1	35.0	0.5
	56	6.5	1.3	41.2	4.8	7.0	1.9	35.0	0.5
	63	3.8	2.0	40.2	5.5	6.0	1.6	38.2	1.3

* Average of five cheeses.

Summary: High concentrations (3-10 ppm) of ozone gave the appearance of destroying heavy mold growth. This was most evident on sides of unwaxed cheese. When the ozone treatment was discontinued a profuse growth of mold developed very rapidly on the cheese, indicating the molds were not destroyed. Slight mold growth developed on the waxed cheese when exposed to high ozone concentrations. Low (0.2-0.3 ppm) ozone concentrations retarded the growth of mold on the sides of unwaxed cheese and aided in preventing mold from developing on the ends.

The sides of the waxed cheese were protected from mold growth while mold continued to grow on the ends. Free mold spores were reduced in numbers in the ozone-treated rooms. The use of ozone did not cause any flavor defects in the cheese.

2.6.7 Sanitizing of Wine Processing Equipment

2.6.7.1 Hampson (2000)

Hampson (2000) points out that since the EPRI Expert Panel's GRAS affirmation (EPRI, 1997), many wineries have embraced the use of ozone for multiple purposes (barrel cleaning and sanitation, tank cleaning and sanitation, cleaning-in-place systems, and for general purpose

sanitation. In the wine industry, ozone systems tend to be mobile, with multiple operations in multiple locations in an individual winery. This makes it important that safety features and ozone management systems be in place and that the system itself be reliable and easy to operate.

The wine industry currently is using ozone generators that are based on corona discharge (CD) or UV technologies for generating ozone. The choice really is a question of how much ozone is needed at a certain gpm water flow rate for a specific application. For CIP, 20 gpm may be desired, necessitating a larger (CD) system, whereas only 10 gpm at a lower ozone concentration (UV system) may provide satisfactory barrel washing.

Ozone requires monitoring in the workplace if used for environmental or equipment sanitation. Reliable ozone monitors are readily available, and the supplier of ozonation equipment should be able to assist with the selection, installation and use of such monitors. A manual containing all the relevant safety information for working with ozonation systems is essential; and it should contain operating instructions for the winery's ozone generating system(s). Workplace monitoring for ozone off-gas should be performed, and records should be maintained to ensure compliance with OSHA's ozone regulations.

When ozone is generated and applied, it is important that the concentration and flow rates be verified, and these should be checked periodically by a technician on some regular schedule or interval (e.g., monthly). All ozone generated should be accounted for by checking for leaks in the system and by proper destruction of any excess ozone. If the ozone is being applied as a gas for the fumigation of a storeroom or cellar, monitoring at the far end of the room and feedback control is desirable. If the ozone is dissolved in water and this water subsequently is used for sanitation, there is always some excess ozone that will not be dissolved in solution.

No ozone mass transfer system is 100% efficient. Excess ozone, or entrained ozone gas, should be "degassed" or separated from the water stream prior to delivery to equipment or the processing environment. This excess ozone also should be destroyed or decomposed back to oxygen before being released back into the atmosphere. Thermocatalytic ozone destruct units are small, efficient, and available for this purpose.

It is not enough to just purchase an ozone generator. The winery also should have maintenance, verification of performance, monitoring and, especially in the case of mobile ozone units, an in-place systems approach that ensures the safe use of ozone in the workplace. Properly used, these ozone sanitizing systems are much safer than chemical (chlorine and caustics) or heat-based sanitizing systems.

One concern is that use of ozone will oxidize equipment and facilities, and this can happen if the materials are incompatible with ozone. Most materials used in food processing are cornpatible. Stainless steel (e.g., 316L) is corroded less by ozone than by chlorine, and common plastics used in food processing generally are resistant, including ECTFE (Halar®), PTFE (Teflon®), PVDF (Kynar®), PVC (rigid, schedule 80 or 40), and silicon tubing and gaskets. Natural rubber will degrade readily; however FPM (Viton®) and Teflon gaskets an very stable.

When ozone is used in high (gas phase) concentrations, stainless steel, Teflon, and Kynar are the best construction materials. PVC should be avoided under high concentration conditions. In

general however, high concentrations (in the low percent range) are only found inside the generator or in the ozone-to-water contacting system. Aside from natural rubber, brass and copper also should be avoided for concentrations over 1.0 ppm of ozone dissolved in water.

Recently at California Polytechnic State University, a study was performed to evaluate ozone's effectiveness as an environmental sanitizer. The fruit and vegetable pilot plant in the university's Food Science and Nutrition Department was the location for this test, and the ozone system used in the study was able to deliver an applied ozone dose of 200 ppm through a 10 gpm hand-held spray wand, typically delivering a residual (measurable) dose of around 1.5 ppm ozone-in-water solution. Environmental ozone monitoring was performed using an ambient monitor, and concentrations in solution were verified using a dissolved ozone monitor.

Various surfaces in the facility were sprayed with the ozonated water in a back and forth fashion for one minute. The test surfaces included a polished stainless steel mixing kettle and table top, stainless steel shroud, central floor drain, a plastic shipping container, and two locations on the non-slip epoxy-coated concrete floor of the facility (area 1 is high-traffic and area 2 is low-traffic). Test areas were not cleaned prior to sanitation, so only the effect of the ozone spray wash was measured. Testing was repeated four times, and microbial load of a 100 cm^2 area was measured before and after ozone application, using both aerobic plate count and bioluminescence. Results are presented in Table 2.6.14.

The results indicate that ozone applied as a spray wash is effective in reducing microbial load in the processing environment. The drain presented problems during the test because the ozonated water applied to the drain washed throughout the long central drain ditch which made results inconclusive. A second test on the drain for two minutes of exposure did provide a reduction in microbial load (see Table 2.6.14).

One advantage ozone has is its ability to readily oxidize microbes in solution. Thus, once a surface is spray-washed, the microorganisms physically lifted from the surface will be killed as they find their way to a drain. The data above represents one series of tests over a two-week period (evaluations performed approximately every third day). With continued or daily use, it is reasonable to expect that the microbial load will be significantly eliminated at all locations exposed to the ozone.

Table 2.6.14. Ozone Sanitation of a Food Processing Facility (Hampson, 2000)

Location	% Reduction (plate Count)	% Reduction (Bioluminescence)
Stainless Steel (kettle)	89.7 to 98.2	87.6 to 91.8
Stainless Steel (table top)	98.9 to 99.7	90.0 to 93.8
Stainless Steel (shroud)	63.1 to 99.9	68.8 to 92.2
Floor Surface, area 1	67.0 to 95.6	75.2 to 96.1
Floor Surface, area 2	84.3 to 99.9	32.8 to 48.8
Floor Drain	*	54.7 to 66.5
Floor Drain (2 minutes)	77.5	92.9 to 99.4
Plastic Shipping Container	96.9 to 97.2	68.9 to 97.4
* Due to drainage and sampling problems, results from this location were inconclusive.		

Because ozone requires no storage or special handling or mixing considerations, it may be viewed as advantageous over other chemical sanitizers. Some may consider the fact that ozone leaves no sanitizing residual a disadvantage, but if a residual is desired, there are many other sanitizers available to accomplish that. Ozone can be considered a complimentary sanitizing regime to help maintain the overall cleanliness and sanitation of wineries or any other food processing facility.

SUMMARY OF MISCELLANEOUS APPLICATIONS SECTION

The efficacy of ozone treatment to achieve improved sanitary conditions has been demonstrated in a wide range of food products and processes in numerous studies from 1960 to 2000. Ozone gas in air and ozone gas dissolved in water have been investigated extensively. Direct contact of ozone with moist microbial cell walls is essential for microbiocidal action. Application methods and procedures must assure direct access to the microorganism surface, an adequate applied dose of ozone, and a finite contact time for effective antimicrobial action. Appropriate conditions are described in great detail in many references cited in the preceding pages.

Microbiologically effective treatments can be applied without significant impairment of food product sensory qualities (appearance, taste, odor, color, texture) and nutrient content (thiamin, riboflavin). Laboratory studies have demonstrated 5-logs or greater count reduction with a wide range of test microorganisms. Commercially treated products typically show count reductions of 2 to 3 logs in actual plant tests.

A wide range of microorganisms, specifically coliform, micrococci, bacilli, yeasts, and molds yield to ozone. Available moisture, humidity, temperature, and competing organic material impact the dose required. Generally, coliform bacteria require the lowest doses of ozone, whereas bacilli and fungi, particularly *Penicillium*, require highest doses of ozone for effective Antimicrobial Action.

3.0 ANALYTICAL METHODS FOR OZONE

In the food industry, ozone is applied in the gas or liquid (aqueous) phases. In the gas phase, ozone is passed into enclosed food storage areas where it suppresses growth of microorganisms. In the aqueous phase, ozone gas is passed into water where some of the ozone dissolves. The aqueous solution containing ozone then is used for washing or otherwise contacting foodstuffs.

Since ozone has a relatively short half-life in water, depending on the presence of ozone-demanding substances in the water, and since its half-life in air is not more than several hours, there is no likelihood of molecular ozone being present in the foods after treatment either in the gas or aqueous phases.

For process control and for safety purposes, however, it is helpful to be able to analyze for ozone in both gas and aqueous phases. In this section are presented five published analytical procedures for measuring ozone. Three of these relate to the aqueous phase and two to the gas phase. Four of the procedures have been published by the International Ozone Association as "IOA Standardized Procedures" and the fifth has appeared in the Food Chemicals Codex.

3.1 ANALYSIS OF OZONE IN THE GAS PHASE

3.1.1 Photometric Measurement of Low Ozone Concentrations in the Gas Phase – IOA Standardisation Committee 008/89(F)

Object: The present standard method concerns the determination of ozone in air, oxygen or other gases in the range below 2 g/m^3 (N.T.P.)

Background: The strong absorption of ozone between 200 and 300 nm (Hartley band) can be used for the determination of ozone concentration in the gas phase. The method is based on the Lambert-Beer absorption law in the form:

$$I = I_0 10^{-Acd}$$

where I and I_0 are the measured UV intensities passing the absorption cell with and without ozone present, c is the ozone concentration in mole per liter, d the internal width of the absorption cell (cm) and A the molar extinction coefficient in L mol^{-1} cm^{-1}.

The value of A is accurately known for the wavelength of the mercury resonance line at 253.7 nm, which is close to the absorption maximum of ozone. Most spectrophotometers are capable of measuring the absorbance or optical density (OD):

$$OD = \log [I_0/I] = Acd$$

from which the ozone concentration c can be derived:

$$c = OD/Ac$$

To obtain the concentration C_N at normal conditions (NTP), the temperature T and pressure P in the absorption cell must be taken into consideration:

$$C_N = \frac{OD}{Ad}\frac{P_N}{P}\frac{T}{T_N}(C_N \cdot is \cdot mole \cdot per \cdot L \cdot - - - \cdot NTP)$$

The normal conditions (NTP) are T_N = 273.15 K; P_N = 1.013 x 10^5 Pa (corresponding to 0EC and 760 Torr or 1.01325 bar). To obtain the concentration in g/m^3 (NTP) the value of C_N has to to be multiplied by the factor 48,000.

The molar absorption cross-section (with log base 10) of ozone at the wavelength 8 = 253.7 nm is:

$$s_{M.10} = 300\ ^{m2}\ mol^{-1}$$

within an uncertainty of less than 1% resulting in a molar extinction coefficient:

$$A = (3000 \forall 30)\ L\ cm^{-1}\ mol^{-1}\ (NTP)$$

Accuracy: To get enough absorption at low ozone concentrations an adequately long absorption cell or folded beam arrangements are recommended. Adequate accuracy can be obtained with double-beam spectrometers with digital electronics. The wavelength should be set to 253.7 nm and the slit width should correspond to a wavelength resolution of about 1 nm. It is also possible to use a low pressure mercury lamp which emits mainly at 253.7 nm in combination with a suitable interference filter to cut out unwanted background illumination. It is also of advantage to use solar blind detection. The accuracy of the apparatus should be checked with calibrated quartz neutral density filters, the optical density of which has been determined at the same wavelength. To avoid ozone destruction a minimum gas flow of at least 1 liter per minute should be maintained in the absorption cell. With adequate instrumentation ozone concentrations can be measured with accuracy better than 5% of maximum scale reading.

Interference: Since the absorption cross-section of ozone at 254 nm is by orders of magnitude larger than those of most other gases, the interference of other air pollutants (nitrogen oxides, carbon monoxide, hydrocarbons, water vapor) can be neglected. Dust and water vapor condensation in the measuring cell must be avoided for reliable measurements.

Mailing Address: IOA-Standardisation Committee, c/o CIBE, 764, Chaussée de Waterloo, B-1180 Brussels, Belgium.

3.1.2 Iodometric Method for the Determination of Ozone in a Process Gas (Revised IOA Standardized Procedure 001/96)

Object: The present standardized method concerns the determination of ozone in air, oxygen or other process gases.

Range of Application: The method is directly applicable in the range of 1 g/m^3 to 200 g/m^3 of ozone, the volume being expressed at NTP (Normal Temperature Pressure, conditions which equal: 0EC or 273.15 K and 1.01325 x 10^5 Pa or 1 Atm).

Reagents (all of analytical grade):

- Quality of the water for make-up of solutions shall comply with ISO No. 3696-1987 Grade 1).

- Buffered KI (potassium iodide) in water:

  KI 20 g/L; $Na_2HPO_4.2H_2O$ (disodium hydrogen phosphate) 7.3 g/L and KH_2PO_4 (monopotassium dihydrogen phosphate) 3.5 g/L.

- Sodium thiosulfate: $Na_2S_2O_3$: 0.1 mol/L in water.

- Acidifying solution: H_2SO_4 (sulfuric acid): 4.5 mol/L

- Powdered KIO_3 (Potassium periodate).

- Crystalline KI.

- HCl (hydrochloric acid) or H_2SO_4: 0.1 N (certified).

- Starch indicator: ZnI_2 (zinc iodide)-starch, prepared by dispersing 4 g starch into an aliquot of water. The dispersion is added to a solution of 20 g $ZnCl_2$ (zinc chloride) in 100 mL water. The solution is boiled until the volume has been reduced to 100 mL and is finally diluted to 1-L while adding 2 g of ZnI_2. The indicator is stable for at least one month when stored in the dark at room temperature.

Standardization of Titrant:

Principle:

$$5\ KI + 5\ H^+ = 5\ HI + 5\ K^+$$

$$KIO_3 + H^+ = HIO_3 + K^+$$

$$HIO_3 + 5\ HI = 3\ I_2 + 3\ H_2O$$

$$3\ I_2 + 6\ (S_2O_3)^{=} = 6\ I^- + 3\ (S_4O_6)^{=}$$

Procedure: To 50 mL of water in a 250 mL conical flask (Erlenmeyer) are added 0.05 g KIO_3 and 0. g KI, followed by another volume of about 50 mL water. After mixing, 10 mL of certified 0.1 N acid are added. The iodine formed is titrated with the thiosulfate solution.

Results: Normality of $(S_2O_3)^{=}$ equals: Normality of acid multiplied by the volume of acid (mL) and divided by the volume of thiosulfate titrant (mL).

Determination of Ozone

Procedure:

- 200 mL of KI solution are added to a gas washing bottle equipped with an open gas bubbling device (tube or diffusor) under a reagent depth of 15 cm or more; (the use of fritted glass diffusors is not recommended).

- A second identical flask is connected in series as a guard detector for ozone transfer and reaction in the first flask.

- Process gas containing ozone is bubbled at a flow rate of 1 L/minute or less, until a total (estimated or expected) quantity of approximately 1 mM O_3 (it equals 0.048 g) has passed.

- The iodine formed in the solutions of KI in the flasks, immediately after acidification with 5 mL of the acidifying reagent is titrated with a freshly standardized sodium thiosulfate solution.

- After titration to a pale yellow color, optionally, 0.5 mL of the starch indicator solution can be added to complete and record the final result. (This addition is recommended, but can be optional, depending on the skill and experience of the operators).

Results:

$$O_3 + 2\ I^- = I_2 + O_2 + O^=$$

$$I_2 + 2\ (S_2O_3)^= = 2\ I^- + (S_4O_6)^=$$

Concentration of ozone in g/L equals: 24 x volume of thiosulfate in L x Normality of thiosulfate divided by the inlet volume of gas passed in L.

Precautions:

- All upstrearn transfer and pressure reducing equipment must be in materials which do not react with ozone, e.g., glass, PTFE . ...

- The gas contacting systems must have a free exit to ambient pressure.

- All gas flow must be expressed at NTP, (for high precision or when analyzing high ozone concentrations, the volume must be corrected for local existing atmospheric pressure).

- Gas flows should be measured with an accuracy of 1%: totalizing volumetric gas meter or with a bubble trap).

Precision And Accuracy:

- Detection limit of the analytical procedure: 0. 1 mg/L

- Repeatability: 2% of the measured ozone concentration.

Interferences: nitrogen oxides, other oxidants of iodide ion, if present.

3.2 ANALYSIS OF OZONE IN THE AQUEOUS PHASE

3.2.1 Colorimetric Method for the Determination of Traces Of Ozone in Water (Indigo-trisulfonate Method) IOA Standardisation Committee 006/89 (F)

Object: The present Standardized Method concerns the determination of traces of ozone in water when other oxidants are absent.

Range of Application: The method is directly applicable in the range of 0.01 to 0.1 mg L^{-1} of ozone in water.

Reagents (all analytical grades):

- **Stock solution** of indigo trisulfonate made up from $C_{16}H_7N_2O_{11}S_3K_3$ (ref.: Riedel de Haën AG-33317) as a 1 mMol L^{-1} solution by dispersing the dye into a solution of analytical grade phosphoric acid at a concentration of 1 x 10^{-3} Mol L^{-1} (Test: A 100-fold dilution of this solution has an absorbance of 0.16 ∀ 0.01 cm^{-1} at 600 nm and should be discarded if the absorbance is lower than 80% of the starting value. Normal stability lasts one month).

- **Diluted reagent:** 20 mL of the stock solution is diluted to 1 liter together with 10 g of analytical grade NaH_2PO_4 and 7 mL concentrated analytical grade H_3PO_4 (stability of the diluted solution: one week).

Procedure:

- 10 mL of diluted reagent solution is introduced into each of two 100 mL volumetric flasks. Fill one flask with ozone-free water (e.g., distilled water). Fill the other flask with the sample water by introducing the sample below the surface of the dye solution to prevent ozone loss by degassing.

- Measure the difference in absorbance at 600 nm between blank and sample with 5- or 10-cm cells. The measurement is to be conducted as soon as possible, but in all instances within 4 hours.

- pH-value of the measured solution must be lower than 4.

Results: The proportionality constant is 0.42 ∀ 0.01 cm^{-1} per mg L^{-1} ozone, which is equal to a difference in absorbance of 20,000 L Mol^{-1} cm^{-1} (stoichiometry is considered as 1 to 1):

$$mg / L \cdot (O_3) = \frac{total \cdot volume \cdot (100 \cdot mL) \cdot x \cdot \Delta \cdot absorption}{cell \cdot length \cdot (cm) \cdot x \cdot 0.42 \cdot x \cdot volume \cdot of \cdot sampled \cdot water \cdot (90 \cdot mL)}$$

Precision and Accuracy:

Detection limit	0.002 mg/L
Accuracy	0.005 mg/L when thermostated cells are used.

Interferences: Other oxidants interfere when used in the treatment of the water. The water must be clear, that means of low turbidity and color.

Remarks: Further reading:

H. Bader & J. Hoigné, Ozone Sci. & Eng., 4, 169-178 (1982).

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3.2.2 Colorimetric Method for the Determination of Residual Ozone in Water (Indigo-trisulfonate Method) IOA Standardisation Committee 004/89 (F)

Object: The present Standardized Method concerns the determination of residual ozone in water when other oxidants are absent.

Range of Application: The method is directly applicable in the range of 0.06 to 0.6 mg ozone per liter in water.

Reagents (all analytical grades):

- **Stock solution** of indigo trisulfonate made up from $C_{16}H_7N_2O_{11}S_3K_3$ (ref.: Riedel de Haën AG-33317) as a 1 mMol L^{-1} solution by dispersing the dye into a solution of analytical grade phosphoric acid at a concentration of 1 x 10^{-3} Mol L^{-1} (Test: A 100-fold dilution of this solution has an absorbance of 0.16 $\forall$ 0.01 cm^{-1} at 600 nm and should be discarded if the absorbance is lower than 80% of the starting value. Normal stability lasts one month).

- **Diluted reagent:** 100 mL of the stock solution is diluted to 1 liter together with 10 g of analytical grade NaH_2PO_4 and 7 mL of the analytical grade H_3PO_4 (stability of the diluted solution: one week).

Procedure:

- 10 mL of diluted reagent solution is introduced into each of two 100 mL volumetric flasks. Fill one flask with ozone-free water (e.g., distilled water). Fill the other flask with the sample water by introducing the sample below the surface of the dye solution to prevent ozone loss by degassing.

- Measure the difference in absorbance at 600 nm between blank and sample with 4- or 5-cm cells. The measurement is to be conducted as soon as possible, but in all instances within 4 hours.

- pH-value of the measured solution must be lower than 4.

Results: The proportionality constant is 0.42 ∀ 0.01 cm^{-1} per mg L^{-1} ozone, which is equal to a difference in absorbance of 20,000 L Mol^{-1} cm^{-1} (stoichiometry is considered as 1 to 1):

$$mg/L \cdot (O_3) = \frac{total \cdot volume \cdot (100 \cdot mL) \cdot x \cdot \Delta \cdot absorption}{cell \cdot length \cdot (cm) \cdot x \cdot 0.42 \cdot x \cdot volume \cdot of \cdot sampled \cdot water \cdot (90 \cdot mL)}$$

Precision and Accuracy:

Detection limit	0.01 mg/L
Accuracy	0.03 mg/L when thermostated cells are used.

Interferences: Other oxidants interfere when used in the treatment of the water. Manganese oxides interfere or must be corrected for. The water must be clear, that means of low turbidity and color.

Remarks: Further reading:

H. Bader & J. Hoigné, Ozone Sci. & Eng., 4, 169-178 (1982).

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3.2.3 Analysis of Ozone in Water – Food Chemicals Codex, 4th Ed., July 1, 1996, p. 277.

Ozone: Triatomic Oxygen: O_3

Formula wt: 47.9982 CAS: [10028-15-6]

Description: Ozone is an unstable, colorless gas with a pungent, characteristic odor. It is produced *in situ* from oxygen either by ultraviolet irradiation of air or by passing a high-voltage discharge through air (oxygen, or mixtures thereof). It is a potent oxidizing agent that decomposes at ambient temperature to molecular oxygen.

Functional Use In Foods: Antimicrobial and disinfectant for water to be used for direct consurnption,. such as for ice. or for indirect consumption, such as for water used in the treatment or display of fish. produce, and other perishable foods. It is also used in the treatment of wastewater.

Requirements:

Identification:

Reagent Solution: Disperse 124.5 mg of alizarin violet 3R in 500 mL of water in a 1-L volumetric flask. Mechanically stir overnight. Add 20 mg of sodium hexametaphosphate, 48.5 g of ammonium chloride, and 6.2 mL of ammonium hydroxide (equivalent to 1.6 g of NH_3).

Dilute to volume with water, and stir overnight. A 10-fold dilution of this solution has an absorbance of 0.155 AU cm^{-1} at 548 nm; the pH of dilutions with sample waters is between 8.1 and 8.5.

Procedure: Introduce 20 mL of the *Reagent Solution* into each of two 200-mL volumetric flasks. Fill one flask with ozone-free water to serve as the blank. Fill the other with the sample by directly introducing the sample, with the aid of a long-stemmed funnel or pipet, below the surface of the *Reagent Solution* to prevent ozone loss by degassing. Immediately measure the absorbance of both solutions at 548 nm, using 1- to 5-cm. cells. The presence of ozone is indicated if the sample solution has a lower absorbance than the blank.

Assay: 0.01 to 0.5 mL; of O_3 per L.

Tests:

Assay:

Indigo Stock Solution: In a 1-L volumetric flask, dissolve 0.770 g of potassium indigotrisulfonate in 500 mL. of water and 1 mL of phosphoric acid, dilute to volume with water, and mix. A 1:100 dilution of this reagent has an absorbance of 0.20 ∀ 0.010 cm^{-1} at 600 nm.

Indigo Reagent I: Just before use, transfer 20 mL of *Indigo Stock Solution*, 10 mL of monobasic sodium phosphate, and 7 mL of phosphoric acid into a 1-L volumetric flask, dilute to volume with water, and mix.

Indigo Reagent II: Proceed as directed for *Indigo Reagent I* using 100 mL of *Indigo Stock Solution* instead of 20 rnL.

Malonic Acid Reagent: Dissolve 5 g of malonic acid in water and dilute to 100 mL.

Procedure (for a concentration range of 0.01 to 0.1 mg of ozone per L): Add 10.0 mL of *Indigo Reagent I* to each of two 100-mL flasks. Fill one flask with ozone-free water to serve as the blank. Fill the other with the sample by directly introducing the sample, with the aid of a long-stemmed funnel or pipet, below the surface of the dye solution to prevent ozone loss by degassing. Without delay, mix and measure the absorbance of each solution at 600 nm, preferably in 10-cm cells. (For a concentration range of 0.05 to 0.5 mg of ozone per L, use *Indigo Reagent II* and proceed as above).

Control of Interferences: In the presence of chlorine, add 1 mL of *Malonic Acid Reagent* to both flasks before adding the samples. Proceed as above, but measure absorbance immediately.

Calculate the concentration of Ozone, in mg/L, by the formula:

$$100D/(f \times b \times V)$$

in which *D* is the difference in absorbance between the sample solution and blank solution; *b* is the path length in cm; *V* is the volume of sample in mL (normally 90 mL); and *f* is 0.42.

4.0 Safety of Ozone

The basic safety aspects of ozone were discussed in Section 1.2.8 (Volume 1). However, from the standpoint of food plant operations, several additional comments should be made. In particular, when treating foods with ozone, not only is the safety of plant personnel of concern, but also the safety of the food(s) being treated with ozone as well as the safety of plant equipment.

4.1 SAFETY OF PLANT PERSONNEL

4.1.1 To Ozone in the Gas Phase

It is axiomatic that plant personnel should not be exposed to ozone in environments where ozone can be inhaled. Although ozone is ***not*** a carcinogen, it ***is*** a very strong oxidizing agent, and is capable of damaging the sensitive tissues in human nasal and lung passages. Consequently, all precautions should be taken in the design and operation of ozone systems in food plants to avoid and/or eliminate exposure of plant workers to gaseous ozone, in particular to levels above the OSHA Permissible Exposure Levels. Therefore, instruments that continuously monitor the ozone gas concentration in the environment should be installed at strategic plant locations where people and ozone could be present. These instruments should be connected to warning devices that will indicate that ozone in excess of the OSHA approved Permissible Exposure Level has been detected.

In closed food storage areas where ozone is applied in the gas phase, plant personnel should be warned not to enter these areas while ozone is being applied. When it becomes necessary for plant personnel to enter such areas, the ozone generating equipment should be turned off and the area ventilated or exhausted for an hour or two so as to remove most or all of the ozone remaining in the atmosphere, or at least to reduce its level to below levels considered safe for plant personnel by the OSHA (see Section 1.2.8.1).

In the event that it becomes necessary for plant personnel to enter enclosed food storage areas which still contain measurable amounts of gas-phase ozone above the OSHA limits, a self-contained breathing apparatus (face mask with self-contained air) should be worn. Such apparatus should be kept outside of the storage area near the entrance door, along with appropriate warnings about the dangers of breathing ozone and instructions for the use of self-contained breathing device.

Under ***no*** circumstances should gas masks be worn which contain activated carbon when entering enclosed areas containing ozone in the gas phase. Although activated carbon readily and rapidly destroys ozone, in so doing, some carbon monoxide and carbon dioxide are produced and will be inhaled by plant personnel wearing such equipment.

4.1.2 To Ozone in the Aqueous Phase

When ozone is applied and used in the aqueous phase, the danger to plant personnel continues to be from exposure to excessive (above OSHA limitations) ozone in the gas phase. This is

because of ozone's limited solubility in water, which results in some ozone escaping the aqueous phase into the surrounding air environment in the plant near the point(s) of ozone application.

On the other hand, there is no danger to plant personnel of exposure to ozone dissolved in water. When water containing dissolved ozone comes into contact with human flesh, the effect is the same type of antiseptic action against microorganisms on the skin surfaces as occurs with other antimicrobial agents (e.g., chlorine and hydrogen peroxide). However, this effect with ozone is much less than those of chlorine and hydrogen peroxide due to its lesser solubility of ozone in water. It is advisable, however, to wear non-latex protective gloves when handling food which has been exposed to freshly ozonated water.

The application of gaseous ozone to aqueous solutions can pose another gas-phase ozone exposure problem to plant personnel, however. When ozone is generated by corona discharge (CD) equipment, the concentration of ozone exiting the CD ozone generator can be as high as several percent (by weight), or several thousand ppm, and every precaution should be taken not to expose plant personnel to these ozone levels. When generated by ultraviolet (UV) radiation, the concentration of ozone generated is only a fraction of that generated by CD – however much less ozone is generated by UV than by CD over a given period of time.

Fortunately for the food industry, relatively new to the use of ozone, this same safety problem exists in drinking water and bottled water plants in which ozone has been used safely for many decades. The proven approach to plant worker safety lies in the appropriate engineering design of ozone generation and application systems coupled with appropriate ozone monitoring equipment. As an example, the contacting of ozone with water in drinking water treatment and bottled water plants is conducted in closed contactors. Any ozone off-gassing the ozone contactor is led (still in an enclosed system) into an ozone-destruction system before the treated off-gas is discharged to the ambient air.

When handled in this manner, plant personnel are never exposed to gas phase ozone and the plant discharges to the local air environment never exceed state, local, or national discharge levels for ambient ozone.

To ensure the proper operation of ozone generators as well as the absence of greater than OSHA-allowed levels of ozone in the plant environment, it is customary in water/bottled water plants to design into the ozone generation equipment a gas-phase ozone monitor that checks the concentration of ozone in the output gas of the ozone generator. This allows plant personnel to know immediately if and when some problem has arisen to cause the ozone output from the generator to fall below rated specifications.

Additionally, it is customary in water/bottled water plants operating with ozone to install one or more ambient air ozone monitors at strategic points within the plant to monitor the level(s) of ozone in plant air. The ozone monitor can be set at, say, 0.1 ppm (the OSHA PEL) or lower. If and when the concentration of ozone exceeds the set level, three responses can be designed into the system:

1. An alarm sounds

2. Exhaust fans can start and remove the ozone-contaminated air

3. Power to the ozone generating system can be ceased, thereby stopping the generation of additional ozone.

Food processing systems that produce ozonated water for aqueous application also can benefit from an ozone monitor in the aqueous stream after the injection point. This alerts plant personnel immediately if and when some problem arises to cause the ozone output from the generator to operate outside of rated specifications. Such a monitor also ensures the maintenance of the appropriate residual ozone level in the water to accomplish its intended treatment objective(s).

After ozone has been applied to waters or wastewaters in a food processing plant, the ozone-containing waters then can be sent to specific point(s) in the food processing plant to wash or otherwise come in contact with specific foods. Again, the hazard to plant personnel lies in the small amount of dissolved ozone that escapes the aqueous phase into the surrounding plant air. For this reason, it is always advisable to conduct such washing operations in a hooded area in the plant, with the ozone-containing air being sent to the ozone destruct equipment, or discharged directly to the local environment provided that the concentration of ozone is below that required by local ambient air regulations.

If hooding is not an option, then low levels of dissolved ozone should be applied in a manner to minimize off gassing and ensure that the amount of ozone escaping to the plant air is always below the OSHA PEL. This approach will ensure that the level of ozone in plant exhaust air will always be below the EPA ambient air standard for ozone.

4.2 SAFETY OF FOOD PRODUCTS TO OZONE

Because ozone is both a strong antimicrobial agent as well as a strong oxidizing agent, it is important for food plant personnel to realize that there is (a) a minimum ozone concentration below which ozone will have little microbiocidal effect and (b) a maximum level for each food product above which ozone is likely to change the appearance of the food due to excessive oxidation. For good plant practice it is recommended that sufficient testing be conducted to establish the minimum and maximum ozone treatment levels so as to achieve (a) the antimicrobial effect desired without (b) changing the nature of the food product. For example, it has been observed that over-ozonating carrots results in some bleaching of the typical orange color (Liew and Prange, 1994).

4.3 SAFETY OF PLANT EQUIPMENT TO OZONE

Hazards to plant equipment arise from the strong oxidizing effects of ozone. Although ozone generators and ozonation equipment (contactors, off-gassing equipment, monitors, etc.) usually are designed by equipment suppliers using ozone-resistant materials of construction, many times ozone generators are retrofitted into existing food plants that use equipment that was not designed to be ozone-resistant.

For example, plant equipment which has parts made of natural rubber and some types of plastics will wear faster if gaseous ozone comes into contact with them. Natural rubber will become

brittle – other materials also will degenerate. The higher the concentration of ozone to which these non-ozone-resistant materials are exposed, the faster they will deteriorate.

This is all the more reason for the food processor installing ozone system(s) to purchase ozone generation and application equipment designed to minimize the escape of ozone into the plant air environment.

4.3.1 Oxygen Safety Considerations

When generating ozone by corona discharge (CD) techniques, it is oftentimes advantageous to send high purity oxygen or oxygen-enriched air through the ozone generator. Higher concentrations of ozone can be produced (up to double) over air-fed CD ozone generators by these approaches. In turn, this means higher rates of ozone mass transfer (of ozone gas into water) and higher concentrations of dissolved ozone in water, resulting in more effective biocidal and oxidation effects.

On the other hand, many materials, once ignited, are known to burn much faster in the presence of oxygen than in air. Consequently food plant personnel using ozone equipment fed with oxygen should take appropriate precautions to minimize leakages of oxygen into the plant.

Additionally, electrical fires in ozone generating equipment are exacerbated when the generators have been fed oxygen (see Pryor and Rice, 2000). Although most modern large-scale ozone generators at municipal water and wastewater treatment plants have been feeding high purity oxygen to produce ozone for more than a decade with no reported accidents or equipment fires, the same cannot be said for all small-scale ozone-generating equipment. The problem can stem from the supplier of low-cost ozone generators who may be using lesser grade materials of construction and perhaps lesser grade electronics.

The prudent food plant purchasing department, when investigating the use of ozone and being offered ozone generating equipment utilizing oxygen, will engage the supplier in detailed discussions about his equipment operating history, and will ask for references to installations of such equipment that can be visited for discussions with plant personnel. Many small ozone generating systems typically used in food plant operations operate effectively with ambient air passed through a pressure swing absorption air dryer, thus avoiding the need for pressurized oxygen. Some available pressure swing air dryers also produce oxygen-enriched air for feeding ozone generators in food processing plants.

5.0 Tolerance Conditions and Proposed Regulation

5.1 TOLERANCE CONDITIONS FOR OZONE

Because of ozone's rather short half-life coupled with its high rate of reactivity when in contact with ozone-demanding materials, such as food surfaces, there is little or no chance for molecular ozone to accummulate in food products treated with ozone. However, as discussed in the sections of this petition dealing with *safety* (Sections 1.2.8 and 4.0), it is important for the safety of food plant personnel and for the safety of the food products themselves, that appropriate attention be paid to applying ozone so as to minimize or prevent exposure of plant personnel to ozone and also to apply only sufficient ozone to the food product(s) being treated to attain the desired antimicrobial effect(s).

As discussed in the two Safety sections of this petition, the OSHA Permissible Exposure Limit for ozone in plant ambient air is 0.1 ppm time-weighted average over an 8-hour day, 5-days/week. That level becomes the ***Tolerance Condition for Ozone in the Gas Phase*.**

The ***Tolerance Condition for Ozone in Aqueous Phases*** then becomes that level of residual ozone that will not allow the OSHA PEL for ozone in the plant ambient air to be exceeded at any point in the plant where workers can be exposed to gas-phase ozone.

5.2 PROPOSED REGULATIONS (OZONE IN GAS AND AQUEOUS PHASES)

OZONE Triatomic Oxygen CAS: [100828-15-6]

In accordance with ¶ 184.1(b)(2), the ingredient is used to treat food within the following specific limitations:

Category of Food	Maximum Residual Level in Food	Functional Use
All Food Categories	**Aqueous Application:** Minimum residual level in process water necessary to accomplish the intended purpose, within limits of solubility in water and in compliance with current OSHA limits in the workplace environment and current EPA constraints against off-gassing to the atmosphere during processing; no ozone residual detected in final product after completion of processing.	Anti-microbial Agent
All Food Categories	**Gaseous Application:** Minimum necessary to accomplish the intended purpose, within the current limits of OSHA in the workplace and the current EPA constraints against off-gassing to the atmosphere.	Anti-microbial Agent

6.0 Environmental Assessment – Request for Categorical Exclusion

The petitioners request a categorical exclusion from the requirement to prepare an EA or an EIS on the following grounds:

1. Ozone will not become an ingredient of foods because of its rapid decomposition when contacting ozone-demanding materials (foods and food surfaces.

2. When generated and applied properly in food processing establishments, any excess ozone generated will be destroyed or diluted to below current regulatory concentrations.

3. Ozone is a substance that occurs naturally in the environment, and its application in food processing plants does not alter the concentration or distribution of the substance, its metabolites, or degradation products in the environment [21 CFR Ch. I (1-4-98 Edition) §25.32 (r)].

4. Ozone currently is being used safely in food processing establishments in the United States at increasing numbers of applications.

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