OZONE AS AN ANTIMICROBIAL AGENT
IN MINIMALLY PROCESSED FOODS

DISSERTATION

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ABSTRACT

In recent years, consumption of fresh vegetables has greatly increased and consumer's demand for fresh or minimally-processed fresh food (MPF) is on the rise. The risk of contamination by spoilage and pathogenic microorganisms, and growth of these contaminants are of concern because most of these products are consumed without thermal processing. In addition, the recent outbreaks of foodborne diseases caused by *Escherichia coli* O157:H7, *Hepatitis A*, and *Campylobacter jejuni* raised serious questions about the safety of minimally processed food.

Chlorine has been used to decrease the microbial load during vegetable processing; however, it can react with organic compounds, resulting in the formation of carcinogenic trihalomethane compounds. Therefore, many studies were done to find suitable alternatives to chlorine. For decades, researchers recognized the germicidal action and oxidizing potential of ozone. Ozone applications in the food industry are mostly related to decontamination of product surfaces and water treatment. Ozone has been used with mixed success to inactivate contaminant microflora on meat, poultry, eggs, fish, fruits, vegetables and dry foods. Additional research is needed to elucidate the kinetics and mechanisms of microbial inactivation by ozone and to optimize its use in food applications.

The objectives of this research were: 1) to study the pattern of inactivation of
selected spoilage (*Pseudomonas fluorescens*, *Lactobacillus mesenteroides*) and pathogenic (*Listeria monocytogenes*, *Escherichia coli* O157:H7) bacteria by ozone; 2) to explore mechanisms of microbial inactivation by ozone; and 3) to optimize the efficiency of ozonation procedures in lettuce processing.

To study microbial inactivation patterns, a batch and three continuous reaction systems were tested. In the batch reactor, all tested microorganisms showed a similar pattern of inactivation. Microbial inactivation occurred immediately after addition of ozone with little change in counts thereafter. The population decreased 2.7 to 7.5 log cfu/mL in 30 seconds, when 1 mg/L ozone was used for the treatment. *E. coli* O157:H7 was the most resistant and *L. monocytogenes* was the least resistant to ozone inactivation. A dose-response model having two segments appeared adequate in describing inactivation of tested microorganisms.

A continuous reaction system, using a membrane filter, was found suitable to study patterns of microbial inactivation as it minimizes the auto-decomposition of ozone during the reaction and thus a uniform concentration of ozone can be applied throughout the treatment. Inactivation kinetics for all the tested microorganisms revealed concave downward curves. *E. coli* O157:H7 was the most resistant and *L. monocytogenes* was the least resistant to ozone inactivation when tested in this continuous reactor. Application of 2.5 mg/L ozone decreased the count by 5 to 6 log_{10} cfu/mL in 40 seconds. A log-log dose-response model described inactivation data appropriately.

Interaction of ozone with microorganisms was elucidated by detecting cell injury and examining treated cells with a scanning electron microscope. When ozone was used
at 0.55 to 1.85 mg/L, 51 to 78% of survivors were injured. The injured population was
greater at intermediate rather than high C\(^t\) (mg/L x sec) values. Among the tested
microorganisms, *P. fluorescens* was the most while *L. monocytogenes* was the least injured
by ozone treatments.

Electron microscopic analysis showed that ozone caused damage to the cellular
structure; this damage was more pronounced in gram-negative than gram-positive bacteria.
When treated with ozone, gram-positive bacteria seemed to lose some mucoid material
outside the cell wall, whereas gram-negative cells tended to collapse and lose cellular
components. Therefore, apparent structural integrity of the cell can not be correlated with
degree of inactivation of microorganisms by ozone.

The feasibility of using ozone to decontaminate a minimally processed product (i.e.,
shredded lettuce) was explored. Different ozonation media and delivery methods were
tested. Ozonation media include ozonated water, bubbled ozone gas into water, and
gaseous ozone. Delivery methods were tested to ensure intimate contact between ozone
and the treated product. Delivery of ozone was facilitated by stirring, sonication and
stomaching. Average decontamination by water washing, ozonated water, bubbling ozone
was 0.9, 1.1, and 1.4 log\(_{10}\) cfu/g, respectively. Bubbling ozone was significantly better than
water treatment and also more efficient way of lettuce disinfection than ozonated water
treatment. Among the delivery methods, high-speed stir was better for the decontamination
of lettuce than other methods. Maximum inactivation of natural microflora (1.9 log cfu/g)
was achieved by bubbling ozone with high-speed stir. Therefore, it was concluded that
bubbling ozone treatment with high-speed stir will be the most efficient and applicable way
for lettuce disinfection.

In conclusion, inactivation studies on bacterial cell suspensions clearly indicate that ozone is a potent antimicrobial agent. Bactericidal action of ozone varies with the microorganism. Ozone at low concentrations damages the outer membrane of gram-negative bacteria and thus cause dramatic changes in the cell structure. Similar concentrations of ozone cause less damage to the cell wall of gram-positive bacteria, but the agent causes intercellular damage and effectively inactivates the cell. Ozone is a less potent antimicrobial agent against microorganisms in food than in pure cell suspensions. Successful application of ozone in food processing depends on developing methods to ensure good contact between this agent and target microorganisms on the treated food.
To my wife and
both sides of my parents
for their love
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Most useful work is not accomplished by chance or by a single individual. Much devotion from many people has been put into this research to get accomplished. I would like to acknowledge and thank all of those to whom I owe even a little material, time and life from my humble heart.

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I give thanks and glory to my Lord, Jesus Christ who was, is and will be gracious and faithful to me.
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FIELDS OF STUDY

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INTRODUCTION

Sanitizers such as hypochlorite solutions, quaternary ammonium compounds and formaldehyde have been used in food processing facilities to control contaminant microorganisms, particularly those causing foodborne diseases. Use of some of sanitizers such as formaldehyde have been prohibited in foods because of the potential health hazards. On the other hand, the need for potent antimicrobial agents has increased in recent years due to increasing disease outbreaks and emergence of new foodborne pathogens. Illnesses that occurred recently from the incidence of *Escherichia coli* O157:H7 in frozen ground beef patties and burgers (CDC, 1997a) and *Hepatitis A* in frozen strawberries (CDC, 1997b) renewed the interest in new effective control measures. Therefore, the food industry is in search of disinfectants that are effective against common and emerging pathogens and safe to use in many specific applications of food processing. One such compound is ozone (O₃) which has been utilized as a sanitizer in many European water treatment plants since the beginning of this century (Gomella, 1972).

In their review about the history of ozone use, Hill and Rice (1982) noted that ozone was first reported by Maurum in 1785. In 1840, a German-Swiss chemist, Schönbein, claimed its occurrence in the ambient atmosphere and named it ozone (Greek derivation "ozein" meaning "to smell"). In 1857, Siemens developed ozone generating tubes, which
is a prototype of the current electric discharge ozonizer. Brodie, in 1872, reported that ozone is a triatomic molecule of oxygen.

Up to the beginning of this century, ozone had been tested for the preservation of food and food ingredients such as milk, meat products, gelatin, casein and albumin (de la Coax, 1904). Hill and Rice (1982) noted that ozone was also applied for the purification and artificial aging of alcoholic beverages including wine and spirits, disinfection of brewing and cider manufacturing facilities, odor control, and medical therapy. However, most of known applications dealt with treatment of drinking water (Bryant, et al. 1992), and municipal and industrial waste water (Stover and Jarnis, 1981). An allotropic modification of oxygen, ozone is a bluish gas with a pungent and characteristic odor. It has a molecular weight of 48, boiling point of -111.9°C, and melting point of -192.7 °C at the atmospheric pressure (Merck Index, 1989). Ozone weighs ca. 0.135 lb/ft³. The oxidation potential of ozone is high (-2.07 v) compared to that of hypochlorous acid (-1.49 v) or chlorine (-1.36 v) (Brady and Humiston, 1978).

There are many advantages of using ozone as a potent oxidizing agent in food and other industries. It is potentially useful in decreasing the microbial load, the level of toxic organic compounds, the chemical oxygen demand (COD) and the biological oxygen demand (BOD) in the environment. Ozone converts many non-biodegradable organic materials into biodegradable forms. The molecule decomposes spontaneously to oxygen, thus using ozone minimizes the accumulation of inorganic waste matter in the environment (Horvath, et al. 1985). High oxidizing power and spontaneous decomposition also make ozone a viable disinfectant for ensuring the microbiological safety and quality of food products.
Ozone use is permitted in many European and Asian countries. In the past, use of ozone in the food and related industries in the United States was limited. It had been used primarily for the removal of iron, manganese, color, or tastes and odors in water (O'Donovan, 1965). In 1982, United States Food and Drug Administration (US-FDA) affirmed that ozone is generally recognized as safe (GRAS), with specific limitations, for use as a disinfectant in bottled water (FDA, 1982). United States Department of Agriculture (USDA) permitted recycling of reconditioned water in poultry chillers (USDA, 1984). Recently, an expert panel in the US self-affirmed ozone as a GRAS substance (Graham, 1997) for broad food applications. US-FDA had no objection to this affirmation.
CHAPTER 1

LITERATURE REVIEW

Generation and decomposition of ozone

Ozone is formed naturally in the stratosphere in small amounts (0.0325ng/L) by the action of solar ultraviolet radiation on oxygen. Small amount of ozone is also formed in the troposphere as a by-product of photochemical reactions between hydrocarbons, oxygen, and nitrogen that are released from automobile exhausts, industries, forests, and volcanic action. However, the gas produced is very unstable and decomposes quickly in the air (Horvath, et al. 1985).

When used in the industry, ozone is usually generated at the point of application and in closed systems. Ozone is produced at low concentrations (0.03 ppm) from oxygen in the air by radiation of wave-length 185 nm, emitted by high transmission ultraviolet lamps (Ewel, 1946). The corona discharge method has been used most widely to produce large amounts of ozone. When a high-voltage alternating current is applied across a discharge gap in the presence of air or oxygen, it excites oxygen electrons and thus induce splitting of oxygen molecules. Atoms from split oxygen combine with other oxygen molecules to form ozone, O₃. Ozone production varies depending on voltage, current frequency,
dielectric material property and thickness, discharge gap, and absolute pressure within the discharge gap. To optimize ozone production, an efficient heat removal system is essential. Dried air is passed through a high voltage current along the discharge gap, thus converting oxygen into ozone at concentrations up to 4% by weight. The use of pure oxygen is recommended over dried air to maximize the yield of ozone. Dried gas is used to minimize the corrosion of metal surfaces due to nitric acid deposits produced from wet gas inside the generator (Rosen, 1972).

In addition to photochemical and electric discharge methods, ozone can be produced by chemical, thermal, chemonuclear, and electrolytic methods (Hovarth, et al. 1985). A new approach in producing ozone has been implemented by Lynitech, Inc (Texas, USA). This is an electrochemical procedure in which water is split into hydrogen and oxygen atoms by electrolysis. Hydrogen molecules are separated from the gas and water mixture and the oxygen atoms combine to form ozone and diatomic oxygen. The manufacturer claims their system produces ozone at concentrations that are 3 to 4 times higher (15 to 20%) than those attainable by corona discharge.

In upper atmosphere, high energy Ultraviolet (UV) irradiation helps degrade ozone molecules. Ozone is converted to oxygen in the process and absorbs the UV energy before it reaches the earth’s surface (Brady and Humiston, 1978). Levy (1971) postulated that the photolysis of ozone to oxygen atoms could lead to the generation of the hydroxyl radical (•OH), a key reactive species during the decomposition process. In addition to UV irradiation, high pH and presence of hydrogen peroxide (H₂O₂) and activated carbon enhance the degradation of ozone (Jans and Holigne, 1998).
Ozone decomposes in solution in a step-wise fashion, producing in turn hydroperoxyl (•HO₂), hydroxyl (•OH) and superoxide (•O₂⁻) radicals (Adler and Hill, 1950; Grimes et al., 1983; Hoigne and Bader, 1975). The hydroxyl radical is an important transient species and chain-propagating radical. The reactivity of ozone is due to the great oxidizing power of these free radicals. According to Hoigne and Bader (1975), the rate constants for reactions of OH radical with many substrates are very high. Hence, these radicals are consumed preferentially by dissolved species before they encounter dispersed particles such as microorganisms. This occurs even when concentrations of molecular solutes are smaller than those of the particles. In many systems, however, OH radicals react with solutes to form secondary intermediates of lower reactivity (for example, peroxy radicals) which may survive until they encounter a dispersed particle. Decomposition of ozone is so rapid in the water phase of foods that its antimicrobial action may take place mainly at the surface (Hoigne and Bader, 1975).

Measurement of ozone

Approximately a dozen analytical methods for the determination of ozone have been proposed: these can be grouped into physical, physicochemical, and chemical methods. Physical methods are based on measuring particular ozone properties, such as the intensity of absorption in the UV, visible, or infra-red (IR) region of the spectrum. The physicochemical methods measure physical effects of ozone reaction with different reagents; such effects include chemiluminescence or heat of the reaction. Chemical methods measure the quantity of the reaction products which are released when ozone reacts with an
appropriate reagent (e.g., KI or HI) or the reduction in the molecular weight of a polymer. These methods vary widely in sensitivity and accuracy. Adler and Hil (1950), for example, pointed out that KI-based methods measure the total oxidizing capacity rather than the amount of ozone only. Therefore, ozone disinfection data should be carefully interpreted, especially when different analytical techniques were used in different inactivation studies.

The indigo method was developed by Bader and Hoigne (1981) and used in numerous ozone-related researches. In this method, ozone adds across the carbon-carbon double bond of sulfonated indigo dye and decolorizes it. The change in absorbance is determined spectrophotometrically. This method is subject to fewer interferences than most of the colorimetric methods and all iodometric procedures (Golden et al., 1988). These authors also stated that only the UV spectrophotometric method should be used for accurate determination of gas-phase ozone.

Antimicrobial action of ozone

Inactivation mechanisms. In spite of numerous studies which were carried out to uncover the inactivation mechanisms by ozone, the precise reactions to cause microbial death after ozone treatment are still not clear. Ozone self-decomposes generating reactive free radicals in aqueous systems. It has been elucidated that the 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 Hoigne, 1983). The same mechanisms may apply to the destructive effect of ozone on bacteria. The oxidative bactericidal power of ozone
in an aqueous system should depend on the mode of the destructive reaction that dominates in the system.

Giese and Christensen (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, which results in a change in cell permeability, eventually leading to lysis.

Bringman (1955) suggested different modes of action by ozone. He concluded that chlorine selectively destroyed certain enzymes, whereas ozone acted as a general protoplastic 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 *Escherichia 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 sulphydryl groups (SH- to S-) in the enzyme is the principal cause of death. Bancroft and Richter (1931) suggested that ozone causes cellular proteins to flocculate. In studies by Prat et al. (1968) and Scott (1975) on the DNA of *E. coli* the pyrimidine bases were modified by ozonation, with thymine being more sensitive to ozone than cytosine and uracil. Ozone caused a more rapid decrease in beta-galactosidase activity in the cytoplasm than alkaline phosphatase activity in the periplasm in *E. coli* (Takamoto et al., 1992). Komanapalli and Lai (1996) exposed 5 ml of *E. coli* K-12 cell suspension to 600 ppm of ozone gas which was bubbled in the medium at a flow
rate of 20 ml/min. They reported that cell viability was unaffected by short-term ozone exposure (1-5 min) at 600 ppm of ozone gas and the intracellular components, protein and DNA, remained intact, but membrane permeability was compromised. With longer exposures, up to 30 min, cell viability decreased, with a progressive degradation of intracellular proteins.

Kim et al. (1980) examined tritiated \( \delta \) bacteriophage and its RNA to elucidate the mechanism of inactivation by 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 breakage. Roy et al. (1981) observed that the damage to the viral nucleic acid is the major cause of the inactivation of poliovirus 1 (Mahoney). Ozone not only damaged viral RNA, polypeptide chains present in the viral protein coat were also altered.

Inhibitory spectrum

Bacteria. Ozone inactivates numerous bacteria, which include gram-negative and gram-positive, and both vegetative cells and spore forms (Table 1.1). Microorganisms inherently vary in sensitivity to ozone, and the physiological state (e.g., the stage of growth) and environmental factors (e.g., RH in atmosphere and medium pH) affect greatly the degree of inactivation of these microorganisms by ozone.

Broadwater et al. (1973) reported that the lethal threshold concentration for the
cells of *Bacillus cereus* was 0.12 mg/L while that for *E. coli* and *B. megaterium* was 0.19 mg/L. The threshold concentration for the spores of *B. cereus* and *B. megaterium* was 2.3 mg/L. Finch et al. (1988) determined the extent of inactivation of *E. coli* using ozone doses of 4.4 to 800 μg/L at contact times of 30 to 120 s. 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 (Montecalvo, et al. 1995). Populations of *P. putrefaciens* decreased by 3 log after 5 min and by 6 log after 20 min of exposure. Addition of glucose (1,000 ppm) to water did not diminish microbial inactivation by ozone, but bentonite (10,000 ppm) decreased the effectiveness of ozone. Dave et al. (1998) showed that a *Salmonella enteritidis* population decreased by 6 log after a short period (30 s) of exposure to a low concentration of ozone (1.5 ppm) in distilled water. When broiler skin contaminated with *Salmonella enteritidis* was exposed to ozone-air mixture (8 %, v/v) for 15 s, about one log reduction in population of the pathogen was observed (Ramirez, et al. 1994). Antimicrobial effects of ozonated water in a recirculating concurrent reactor were evaluated (Restaino, et al. 1995). Death rates among the Gram-negative bacteria (*S. typhimurium, E. coli, P. aeruginosa* and *Yersinia enterocolitica*) were not significantly different, whilst among Gram-positive bacteria, *Listeria monocytogenes* was significantly more sensitive than either *Staphylococcus aureus* or *Enterococcus faecalis*. In a recent study, inactivation of *E. coli* with ozone was done in a semi-batch and continuous flow reactors at different pHs and temperatures and with a radical scavenger (Hunt and Marinas, 1997). They found that *E. coli* was inactivated primarily by molecular ozone.
Foegeding (1985) studied the inactivation of *Bacillus* and *Clostridium* spores and reported that ozone was a rapid and effective sporicide and acidic pH enhanced the lethality. The author also suggested that the spore coat is a primary protective barrier against ozone. Naitoh (1992 a & c) found that the addition of metallozoites, ascorbic acid, and isoascorbic acid improved the inactivation of *Bacillus subtilis* spores by ozone at 5 to 50 ppm for 1 to 6 h. Naitoh (1992 c) also investigated synergistic sporicidal activities of gaseous ozone and UV irradiation. The author reported that combined treatments reduced contact time required for the inactivation.

Fungi. Ozone is also an effective fungicide (Table 1.2). Although the effectiveness of ozone may vary among species, researchers showed that germicidal effect was generally greater for molds than bacteria. Ewell (1938) stated that depending on the cleanliness, a minimum continuous concentrations of 0.6 - 1.5 ppm of ozone is necessary to prevent mold growth on eggs kept at 0.6°C and 90% RH and the equilibrium concentration for beef storage under similar conditions should be between 2.5 to 3.0 ppm. According to Farooq & Ahsaqui (1983), ozone also inactivated yeast. Population of *Candida parapsilosis* decreased by 2 log in 1.67 min when the yeast was exposed to 0.23 to 0.26 mg/L ozone. Naitoh and Shiga (1982) demonstrated that the microbicidal activity of aqueous ozone (0.3 - 0.5 mg/L) against spores of *Aspergillus*, *Penicillium*, and *Candida paraceps* was observed after 90 to 180, 45 to 60, and 5 to 10 min of exposure, respectively. The microbicidal effect of ozone was greater at lower temperature and pH and with addition of 1 to 5% NaCl, however, effectiveness of ozone decrease with the addition of 1 to 10% of
sucrose. In the presence of wheat flour, the microbicidal effect was enhanced against molds and depressed against yeast. Counts of *Candida tropicalis* decreased by 2 log when the yeast cells were exposed to ozone at 0.02 mg/L for 20 s or at 1 mg/L for 5 s (Kawamura et al., 1986). Naitoh (1992b) investigated susceptibility of different yeasts to ozone at different temperature and humidity. *Hansemula anomala*, *Saccharomyces rosei*, *Pichia farinosa*, *Candida parapsilosis*, *Kluyveromyces marxianus*, and *Debaryomyces hansenii* var. *hansenii* were treated with gaseous ozone at 4 to 5 ppm for 1 to 5 h at 30 to 60°C and RH 25 to 90%. At lower temperature and 5 h exposure, counts of *C. parapsilosis* and *K. marxianus* decreased more than one log, however, counts of the other yeasts did not decrease appreciably. 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. More than 4.5 log of *Candida 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 was killed after a 5 min exposure (Restaino, et al. 1995). The average ozone output levels in the deionized water was 0.188 mg/L.

Viruses. Ozone is potentially an effective virucidal agent (Table 1.3). Relatively low concentration of ozone and short contact time are sufficient to inactivate viruses. However, inactivation of viruses in wastewater required longer contact time and larger ozone concentration than inactivation in ozone demand-free systems because of oxidizable materials present in the medium. Majumda et al. (1973) reported a rapid decrease in virus
survival at ca. 1 mg/L initial ozone concentration after 2 min contact period. Kattan 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. Two log of poliovirus type 1 were inactivated with 0.25 mg/L of ozone for 5 min (Harakeh and Butler, 1985), however, only 38% was killed with 0.4 mg/L of chlorine for 15 min (Alvarez and O'Brien, 1982). Although chlorine is the most widely used agent for disinfecting water, its use presents problems because of its relatively low effectiveness as a viricide. Chen and Vaughn (1990) applied chlorine, chlorine dioxide, and ozone for the treatment of human and simian rotaviruses and concluded that ozone is superior to the other two agents. Herbold et al. (1989) tested the resistance of five viruses and bacteria to ozone in steadily flowing water at 20°C and pH 7. The order of resistance was poliovirus type 1 (PV 1) < E. coli < hepatitis A virus (HAV) < Legionella pneumophila serogroup 6 < B. subtilis spores. For the complete inactivation of PV1 and HAV (ca. 10^6 TCID₅₀/mL), 0.13 and 0.25 to 0.38 mg/L ozone was needed, respectively. Emerson et al. (1982) tested ozone to disinfect human epithelial cells (Hep-2) 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/L, respectively, for 30 seconds. Unassociated viruses in the control treatment were inactivated by 0.081 mg/L for 10 s. 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/L and ozone residual of 4.7 mg/L, whereas unassociated viruses was completely inactivated after 5 min with 4.82 mg/L and ozone residual 2.18 mg/L.
Emerson et al. (1982) concluded that viruses associated with cells or cell fragments are protected from inactivation by ozone at concentrations that readily inactivate purified virus.

Protozoa. Table 1.4 lists results of studies on inactivation of some protozoa by ozone. Wickramanayake (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. Two log decrease of population was observed with 0.2 mg/L ozone at 25°C and pH 7 in 7.5 min for N. gruberi compared to 1.05 min for G. muris. The intestinal parasite, Cryptosporidium parvum, which may cause gastroenteric disease, was exposed to ozone which inactivated >90% of the parasite population within 1 min at 1 mg/L ozone in ozone demand-free water (Kurich et al., 1990).

Environmental Factors

Susceptibility of microorganisms to ozone may vary according to the pH of the medium, temperature, humidity (when cells are treated in air), additives (e.g., acids, surfactants, and sugars), and the amount of organic matter surrounding the cells.

Temperature. A decrease in the temperature of an aqueous medium results in increased solubility of ozone. Ozone decomposition, on the other hand, is accelerated with rising temperature. Herhold et al. (1989) reported that ozone effectiveness on HAV and E. coli diminished as temperature rose. However, Katzenelson et al. (1974) indicated that lowering the temperature from 5°C to 1°C had little effect on the inactivation kinetics of
microorganisms, contrary to that reported for chlorine.

pH Medium pH influences the stability and effectiveness of sanitizers. Distribution of free chlorine species, which have different disinfection efficiencies, is governed by the solution pH. However, ozone does not undergo dissociation in water as does chlorine. The stability of aqueous ozone, however, can be improved by decreasing the pH. Leiguarda et al. (1949) reported that bactericidal efficiency of ozone on *E. coli* and *Clostridium perfringens* was slightly greater at pH 6.0 than at pH 8.0. Nutrient broth treated with ozone will support little or no bacterial growth, due to change of pH. Farooq et al. (1977) noted higher survival rate of *Mycobacterium fortuitum* during ozone treatment when pH was increased. The authors attributed this increased survival to smaller ozone residual as pH of the media increased.

Fogedting (1985) studied ozone inactivation of *Bacillus* and *Clostridium* spores at different pH values. This researcher found that acidic pH values enhanced the lethality of ozone. It has been reported that the ozone decomposition rate is more rapid in aqueous solution at high pH due to the catalytic activity of the hydroxyl ion (Adler and Hill, 1950; Hewes and Davison, 1973).

Humidity. Elford and Endo (1942) used low ozone concentrations and long exposures at variable relative humidities to disinfect airborne microorganisms. At relative humidity below 45%, the germicidal power of ozone against air-borne microorganisms was negligible. Inactivation was substantial even for concentrations far below 0.1 mg/L when
high humidities were used. Ewell (1946) demonstrated that microorganisms were killed more readily by ozone in an atmosphere having a high than low relative humidity. The need for moisture in a cell to be inactivated by ozone was elucidated by Guerin (1963), who showed that not only are 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. The author concluded that ozone is an effective inhibitor but only for non-dehydrated microorganisms. Kim and Yousef (unpublished data) found similar reaction of ozone in dried food ingredients containing natural contaminants. They treated a solid food ingredient, having various water activity (w), with gaseous ozone. When w, of the ingredient was ca. 0.95, 10^2 - 10^3 cfu/g were inactivated with 200 ppm ozone in ozone-oxygen mixture. However, a similar ozone concentration had no effect on the microbial load of products with w less than 0.85. In order to counteract this microbial resistance to ozone, water was added to the food ingredients and the mixture was shaken by an orbital shaker at 25°C overnight. This treatment increased w from 0.85 to 0.96 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 used.

Ozone demand of the medium. Having high oxidation potential, ozone reacts with microorganisms fast, resulting in high lethality. However, ozone also reacts with other particles and compounds, if placed in an environment such as food systems which are rich in organic matters.
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 effect the inactivation may follow. Yang and Chen (1979) reported that the bactericidal effects of ozone decreased in Ringer solution, 5% NaCl solution and in the presence of egg albumin in solution. 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 S. 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 (BSA). Residual ozone in water containing BSA 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, which immediately react with solutes. These different pathways of reactions lead to different oxidation products, and they are controlled by different types of kinetics (Stachelin and Hoigne, 1985). The solutes present in water influence appreciably the rate of the radical-type chain reaction leading to the decomposition of ozone. This reaction is promoted by solutes, such
as formic acid and methanol, that convert the nonselective hydroxyl (·OH) into a superoxide (·O\textsubscript{2}·) radical which is a more efficient chain carrier. Such promoters counteract the inhibiting effects of OH radical scavengers which generally terminate the chain reaction. Acetic acid and acetate are known to terminate the reaction by scavenging OH radicals, thus stabilizing ozone in aqueous solutions (Forni et al. 1982; Hoigne 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 direct mode of reaction by ozone predominated at high glucose concentration, however, the OH radical 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, specially when they are present in food systems. The association of microorganisms or sub-cellular components with suspended matter may hamper the accessibility 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 break down clumps of microorganisms and thus increased the antimicrobial effect of ozone dramatically. Barleson 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 Youssef (1998) could not confirm the effectiveness of sonication during treatment of fresh lettuce with ozone. Sonication may enhance the decomposition of ozone itself or increase ozone demand by detaching organic materials from the cut surfaces of the shredded lettuce.

Ozone as an alternative sanitizer to chlorine

Merits and Drawbacks of Chlorine in Food Processing. Chlorine in various forms, especially hypochlorite salts, have been successfully used to sanitize utensils and equipment in dairy and other food processing industries. Hypochlorites are considered GRAS substances and thus are permitted in numerous food applications in the United States. Chlorine compounds are effective and inexpensive disinfectants. For example, use of hypochlorite dips or sprays is effective for controlling bacterial contamination of fruits and vegetables. In the egg production industry, chlorine compounds are used in wash water to decontaminate spoilage and pathogenic microorganisms.

Chlorine compounds have a few drawbacks which increasingly limit its use in the food industry. Chlorination may lead to the formation of toxic or carcinogenic chlorinated organic compounds in water (Brungs, 1973; Page et al., 1976), and food, or on food contact surfaces (Wei et al., 1985). Collins and Deaver (1973) reported that chlorine residues greater than 0.1 mg/L may be excessive with respect to toxicity and that in critical areas of biological significance, it may be necessary to provide dechlorination facilities to reduce chlorine concentration. The recognition of the potentially hazard from the presence
of carcinogenic trihalomethane compounds (THMs) in drinking water, which are formed by the reaction of free chlorine (HOCl, OCl-) with soluble organic compounds, prompted a regulation that sets the maximum level for total THMs at 100 μg/L (CFR, 1976).

It has been reported that chlorine is not very effective against some microorganisms. Brackett (1987) reported that L. monocytogenes on Brussels sprouts was remarkably resistant to chlorine treatment; count of the pathogen decreased by ca. 2 log only with 200 mg/L of chlorine treatment. Beuchat and Bracken (1990) reported that chlorine pre-treatment did not prevent the growth of L. monocytogenes on shredded lettuce when treated product was packaged in modified atmosphere.

In an effort to control or reduce both the hazardous microorganisms and THM levels in potable water, alternative treatment measures have been proposed. These include pretreatment of water to reduce levels of precursor organic compounds, removal of THMs after chlorination, and application of alternative disinfectants, such as ozone, that do not generate THM (Brodmann and Russo, 1979).

Ozone as a chlorine alternative. Much information attesting to the superiority of ozone over other chemical disinfectants have been accumulated. Gomelia (1972) reported that ozone, compared to chlorine, showed stronger and more rapid antimicrobial action against spores, fecal, and pathogenic microorganisms and viruses, mainly in an environment with a high organic-matter content. Kessel et al. (1943) showed that free ozone residues of 0.05 to 0.45 mg/L were sufficient to inactivate poliovirus within two min, while free chlorine residues of 0.5 to 1.0 mg/L at pH 6.0 required 1.5 to 2.0 h for similar degree of
virus inactivation. Another study by Scarpino and his colleagues (1972) also confirmed that ozone is superior to chlorine in the rate of disinfection of poliovirus. With 0.3 mg/L of disinfectant, ozone reduced virus particle count by 2 log within 10 s, while chlorine reduced the count by 2 log in 100 s.

Korich et al. (1990) reported that chlorine dioxide and ozone were more effective than chlorine and monochloramine against Cryptosporidium parvum oocysts. Greater than 90% inactivation of oocysts was achieved with exposure to 1 mg/L ozone for 5 min. Exposure to 1.3 mg/L chlorine dioxide yielded 90% inactivation after 1 h, while 80 mg/L chlorine and 40 mg/L monochloramine required approximately 90 min for 90% inactivation.

Forsythe and Waldroup (1994) reported the economic benefits of ozone usage in poultry processing plants such as reduced water purchase, reduced sewer treatment costs, and savings in electrical energy from recycling ozonated water. With ozone use, for a plant processing 1.3 to 1.5 million broilers a week, weekly savings is expected to be at least $6,000 compared to the use of water without any antimicrobial treatment. In addition to the economic benefits of water recycling, the use of water with fewer chemical residuals will be favorable to the environment.

Limitations of ozone

Reactivity. An often cited disadvantage of using ozone as a disinfectant is that, unlike chlorine, it is extremely unstable (Meadows-Taylor, 1947). It is difficult to predict how ozone reacts in the presence of organic matter. It can either oxidize the compound,
ionize the compound, or spontaneously decompose to oxygen and free radicals. The mechanism of decomposition of ozone is also a complex process which depends on factors such as the types of radicals formed in solution and various types of organic matter present in the medium that either initiate, promote, or inhibit the radical chain reaction. Therefore, it may be difficult to generalize that a particular concentration of ozone at a given rate will always be effective in inhibiting a definite concentration of microorganisms in a food product. After treatment with 1.5 mg/L ozone, water samples with no detectable residual ozone were found to remain sterile for greater than 1 month (Stalder and Klosterkoetter, 1976). However, during passage through a pipeline 1200 m long, recontamination and considerable growth of microorganisms were observed. On inoculating water sterilized by ozone with a normal population of water bacteria, growth was more pronounced than in similar experiments with heat-sterilized water of the same origin. This may indicate that the breakdown products of organic water contaminants (e.g. humic acid) produced during ozone treatment are better nutrients for water bacteria than the original organic substances themselves.

Toxicity. Ozone is a very reactive oxidant which can affect biological systems and interact with cellular components (Lee et al., 1983). Thus, ozone may have toxicity to humans and small animals; this toxicity depends on the concentration and exposure time and is also influenced by genetic and host factors such as age and sex (Mehlman and Borok, 1987). Even though the precise mechanism of cellular damage by ozone is still unclear, the most probable mechanism is free radical reaction in the processes of peroxidation of
polyunsaturated fatty acids (Mudd and Freeman, 1977; Pryor et al., 1983) and oxidation of proteins, amines, and thiols (Mudd et al., 1969). It is common among individuals to experience headache and irritation of the throat, nose, and eyes upon continued exposure to concentrations above 0.1 ppm (Stockinger, 1959). Therefore, the toxic effects of ozone are manifested in the lung upon ozone inhalation and absorption in the lung. While the respiratory tract is the primary target for ozone toxicity, a variety of extrapulmonary damage may result from the ozone and its reaction products (Goldstein, 1979; Borek and Mehlman, 1981).

The concentration of ozone which kills bacteria has been variously reported to be 0.02 to 2.29 mg/L (Table 1.1-1.4), whereas the toxicity for small animals is 3 to 12 mg/L (Stockinger, 1959). In spite of ozone's pleasant odor at low concentrations, 0.1 mg/L is objectionable to all normal humans because of irritation in the nose, throat, and eyes (Witheridge and Yaglou, 1939). Scott and Lester (1963) reported as little as 0.02 to 0.04 mg/L can be detected by man and prolonged exposure to a concentration of or greater than 1,000 mg/L can cause death. Thorp (1950) plotted limiting values for physiological effects of ozone exposure on man. The author indicated that with an hour exposure symptomatic, irritant, toxic, and irreversible lethal effects can be induced by ozone concentration of 2, 4, 15, and 95 ppm, respectively.

Davis (1959) showed that ozone may have a mutagenic effect on E. coli. The author suggested that some of the mutagenic effects of UV irradiation is attributed to the ozone produced by the shorter wavelengths. Hamelin and Chung (1975) reported an increased mutation rate in E. coli exposed to ozone at as low as 0.05 ppm for 5 min,
however, the mutants were sensitive to ozone. Mutagenic effects of ozone have also been suggested in plant (Fetner, 1958) and animal studies (Stockinger, 1962; Brinkman et al., 1964). However, the supposition that ozone is mutagenic or carcinogenic in man may be questionable if it is based primarily on information on the biochemical mechanism of ozone toxicity and on in vitro and animal studies. Noot et al. (1989) reviewed use of the Ames Salmonella assay for assessing the mutagenicity of water produced by various treatment processes. They reported that ozone produced water that is less mutagenically active, and that in the determination of ozone treated water mutagenicity, raw water characteristics, ozone dosage and contact time may be important. Mutagenicity (determined using the Ames test with Salmonella strains TA 100 and TA 98) was observed in chlorinated water but not in water subjected to ozone treatment only (Zhurkov, et al. 1997). Levels of mutagens in chlorinated water could be effectively reduced by subsequent treatment with ozone at 0.5 mg/L. Mutagenic activity of nitro and carbonyl groups formed as disinfection by-products in drinking water was studied using an in vitro chromosomal aberration test (Itoh and Matsuoka, 1996). The authors reported that activity inducing aberrations of chlorinated water reached about 10 times that of waters treated with chlorine dioxide, chloramine and ozone. Disinfection with ozone prior to treatment with either chlorine or monochloramine resulted in a lower level of mutagenicity than when either disinfectant was used alone (Patterson, et al. 1995).

In practical application of ozone in the food industry, safety for the user is an important issue. Ozone detecting systems, respirators and destruction systems are needed for the safety of workers in food processing facilities. In addition, the efficient ozone
treatment for the specific application needs to be developed in order to avoid excess of ozone use. Good manufacturing practice (GMP) and hazard analysis and critical control point (HACCP) systems are also needed to control high ozone demand materials in food processing. Razumovskii and Zaikov (1984) indicated the maximum permissible concentration inside buildings is fixed at 0.1 mg/m² (0.047 ppm). The Occupational Health and Safety Administration (OSHA) worker safety limit is 0.1 ppm on an 8-hour, time-weighted average (CFR, 1997a).

Applications of ozone in food processing

Meat. Ewell (1946) noted that 0.5 and 3 mg/L ozone gas was needed for 50% killing of E. coli on a meat surface. Kaess and Weidemann (1968) sprayed fresh beef with Pseudomonas, Candida scottii, Thamnidium, and Penicillium and then exposed to 0.15 to 5 μg/L of gaseous ozone. They reported that count of non-pigmented Pseudomonas and C. scottii decreased significantly at >2 μg/L ozone and the lag phase of two molds increased but the growth rate didn't change. The color of muscle surface treated with <0.6 μg/L ozone did not differ from that of controls. Ozone also has been applied in the process of tenderizing meats to control surface microflora (Pseudomonas spp., spores, Salmonella spp., Staphylococcus spp.). Ozone in gas mixture at 0.1 mg/L and a relative humidity of 60 to 90% were required in the tenderizing room to inactivate bacteria but higher concentrations of ozone were required to inhibit molds. Kaess and Weidemann (1973) also reported that simultaneous use of UV (0.2 μW/cm²) and ozone (0.5 μg/L) produced
synergistic effects as manifested in an increase of lag phase and decrease of growth rate with molds such as \textit{Thamnidium} spp. and \textit{Penicillium} spp. but not with bacteria such as \textit{Pseudomonas} spp. Fourmaud and Lauret (1972) showed little reduction in counts of \textit{Microbacterium thermosphactum}, \textit{Lactobacillus}, \textit{P. fluorescens}, and \textit{Lactococcus} on the beef surface as a result of gaseous ozone treatment (100 ppm) for 30 min. They concluded that low activity and side effects such as discoloration and odor development rendered ozone use unacceptable. According to Billon (1978), ozone exerted only a bacteriostatic effect against \textit{B. subtilis} or \textit{Micrococcus luteus} grown on agar plates, but a bactericidal effect against \textit{Salmonella}, \textit{S. aureus} or \textit{E. coli}. Trials to use ozone for sterilizing the interior of meat-transport vehicles reduced the counts of aerobic mesophiles, coliforms and sulphite reducing clostridia. Studies on meat also showed that ozone treatment improved the keeping quality, and decreased counts of mesophilic aerobes, and sulphite-reducing anaerobes. Billon (1978) concluded that ozone treatment may be of value for preservation of meat products, and for sanitization of vehicles, and food processing equipments. Ozone at 10-20 \(\mu\text{g/L}\) inhibited the microbial growth on beef kept at 0.4\(^\circ\text{C}\) and 85 to 90\% RH and extended the permissible storage period by 30 to 40\% (Kolodysznaya and Suponina, 1975).

Rau and Krenmer (1989) used ozone for the treatment of air-borne microorganisms on the surface of meat stored at 2.5 to 6\(^\circ\text{C}\) and 92 to 95\% RH and halted the growth of \textit{Enterobacteriacea} but not that of \textit{Pseudomonas}. Green and Jones (1989) studied effects of ozone on beef carcass quality. Beef carcasses were continuously ozonated (0.03 ppm) under 95 \% RH and 1.6 \(^\circ\text{C}\) for up to 9 days of ageing. Although ozone prevented bacterial growth on carcass surfaces, it did not increase the retail case life.
(as judged by odor and appearance) nor reduced bacterial growth on retail steaks. Dondo, et al. (1992) evaluated ozone usage for beef storage in a refrigerator. Ozone stopped the growth of surface contaminants of beef during several days of refrigerated storage and improved sensory quality by decreasing the formation of total volatile N compounds. Spraying with hydrogen peroxide (50 g/L) solution and ozonated water (5 g/L) were more effective to reduce bacterial contamination on beef brisket fat than that with trisodium phosphate (120 g/L), acetic acid (20 g/L) and a commercial sanitizer (3 g/L) when applied after washing with plain water (Gorman, et al. 1995). Reagan et al. (1996) conducted a study to compare procedures and interventions for removing physical and bacterial contamination from beef carcasses. Ozone reduced aerobic plate counts by 1.3 log cfu/cm² which was approximately equivalent to conventional washing in reducing bacterial populations on beef.

Horvath et al. (1985) generalized that in the presence of ozone, the growth of the surface microflora on meat surfaces decreases at refrigeration condition; however, no effect is exerted if the extent of contamination is very large already. Decontamination of meat and poultry carcasses by ozone can reduce human foodborne infections (Bolder, 1997), however, additional control measures and good manufacturing practices should never be neglected.

Poultry. Ozone has been effective in disinfecting hatchery, hatching eggs, poultry chill water and poultry carcass. Sheldon and Brown (1986) evaluated the effects of ozone on the quality of poultry chiller water and broiler carcasses. Carcasses, chilled in tap water
containing ozone at 3.0 to 4.5 ppm for 45 min, were consistently lower in microbial count during storage when compared with non-treated ones. Ozoneation of chilled water decreased microbial load > 2 log and chemical oxygen demand (COD) approximately 33 % and increased light transmission (at 500 nm) without significantly changing the sensory quality of poultry meat.

Cultures of Staphylococcus, Streptococcus, and Bacillus species previously isolated from poultry hatcheries and culture collections of E. coli, P. fluorescens, and S. typhimurium, Proteus species, and A. fumigatus were spread-plated on open petri plates and independently exposed to ozone gas in a prototype laboratory poultry setter (Whistler and Sheldon, 1989b). Ozone treatment (1.5 - 1.65 %, w/w) decreased microbial populations by >4 to 7 log for bacteria and >4 log in case of fungi. Whistler and Sheldon (1989a) also evaluated ozone as a disinfectant against natural contaminants on hatching eggs. Microbial counts were significantly decreased (>2.5 log) from the shell surface of water-misted and ozonated eggs (2.83 %, w/w) for 2 h. However, hatchability was significantly reduced (26.5 - 37.5 %) following ozonation using 3.03 % ozone (w/w) for 2 h. Bailey et al. (1996) concluded that ozone decreased the aerobic plate counts and Salmonella in hatching cabinet air samples by 75 to 99 %. In a different study, eggs were treated with ozone gas (10 - 12 µg/L air) for 6 h, and then stored for 6 months at -1 °C with 86 % RH and 29 °C with 75 % RH (Rudavskaya and Tishchenko, 1978). Eggs were analyzed at various intervals for general sensory quality, changes in acid, peroxide and thiobarbituric acid (TBA) values of the yolk, white and yolk indices, and variations in quality grading. All quality parameters had better values in the ozone treated samples than
in the controls, and that the lower storage temperature had an additional beneficial effect on quality. Krivopishin et al. (1977) suggested a method for preservation of eggs using ozone. Eggs are dipped in paraffin wax at 40 to 45 °C and are then treated for 10 to 30 min in air containing 1 to 3 mg/L ozone. Cox et al. (1995) patented a "Hyperpasteurization" process that involves treatment of shell washed eggs with heat (59.4 °C) and ozone in a vacuum chamber. The treated eggs had an extended shelf life and reduced microbial load.

Yang and Chen (1979) treated broiler parts in ice cold water with gaseous ozone at 3.88 mg/L for 20 min and also disinfected a microbial suspension obtained from fresh and spoiled chicken necks with gaseous ozone at 2.48 mg/L for 5 to 9 min, respectively. They found that the total microbial counts of broiler, fresh and spoiled microbial suspensions were reduced 1, 0.6, 3 log, respectively. They also noticed that ozone treatment preferentially destroyed gram-negative rods. In another study, ozone was used to disinfect microorganisms on poultry meat (Kim and Kim, 1991). All microbial contaminants were inactivated when meat was flushed for 50 min with a gas mixture containing ozone flowing at 1,500 ppm/min. Ozone as one of intervention strategies has been tested and evaluated for controlling pathogenic bacteria including *Salmonella* spp., *E. coli*, and *Campylobacter* spp. during broiler processing (Anon. 1994a).

**Fruits and Vegetables.** Bazarova (1982) stored apples in a specially constructed stainless steel chamber at 0 to 1 °C and 90 to 95 % RH with ozone gas being admitted daily for 4 h at 5 to 6 μg/L. The author concluded that ozone treatment reduced weight loss and spoilage incidence in apples. The oxidation of ethylene and removal of other metabolic products was also observed.
products by ozone also increased shelf life of apples and oranges (Horvath et al. 1985). Ong et al. (1996) examined the effectiveness of chlorinated and ozonated water dips in the dissipation of pesticide (azinophos-methyl, captan, and formetanate hydrochloride) in solution, and on fresh and processed apples. Both treatment decreased pesticides in solution and on apples. However, ozone wash at 0.25 mg/L was not as effective as chlorine wash at 50 and 500 mg/L because of the low ozone concentration and instability and the high organic content of the wash water.

Ozone at 0.1 to 0.3 ppm in the atmosphere during blackberry storage suppressed fungal development for 12 days at 2 °C and did not cause observable injury or defects (Barth et al. 1995). The authors concluded that storage of blackberries in the presence of ozone extended the market life of this perishable product. Kute, et al. (1995) treated strawberry tissue with ozone at 0.3 to 0.7 ppm and stored the product at 2 °C. After 1 wk storage, ascorbic acid levels in ozone-treated fruits was not significantly different from controls, whilst total soluble solids reached significantly greater levels compared to controls. In another study, ca. 65 ppb ozone was applied to strawberries in closed chambers for five days per week for three months. Fruit yield and winter survival were not significantly affected by ozone (Keutgen and Lenz, 1997).

The stabilizing effect of an electric current (applied for 1 h at 0.05 - 0.1 A/cm and 20 v) on carbonation clarified juice was studied (Tedostkin and Zharik, 1978). Microorganisms were not detected after 2 yr of storage. The authors argued that bactericidal action noticed in treated juice was partially due to the formation of ozone during the electric treatment.
Grapes exposed for 20 min to ozone (5 mg/L) had considerably reduced counts of bacteria, fungi and yeasts (Sarig, et al. 1996). Fungal decay following cold storage of the grapes was reduced and shelf life increased by the ozone treatment. Peach trees were exposed to ozone fumes for 43 days in open-top chambers (Badiani, et al. 1996). With increasing ozone levels, surface overcolor and juice pH increased but flesh firmness decreased.

In vegetables, the advantages of ozone were similar to those experienced in fruit processing. Onions and potatoes were stored in wooden chambers covered with polyethylene film in which ozone (0.2 µg/L) was produced for 8 h/day on 5 days/week (Fäiselberg-Blank et al., 1979). Ozone treatment decreased chemiluminescence, oxygen uptake, catalase, and peroxidase activities and had a marked inhibitory effect on growth of surface microorganisms. Losses due to spoilage at the end of storage were 1 and 0.8 % respectively for treated onions and potatoes versus 9.7 and 6.7 % for controls. Baranovskaya et al. (1979) used ozone in the industrial storage of potatoes, onions, and sugar beets. They maintained ozone concentration at 3 mg/L with temperature within 6 to 14 °C and RH at 93 to 97 %. Their analysis showed that bacteria and mold counts were very low for treated samples, and chemical composition and sensory quality didn’t change appreciably. Potatoes stored in the ozone-containing environment (10-20 µg/L) exhibited a 3 to 5 % higher content of starch, 1.3 to 1.5 times lower content of total sugars, and 1.2-fold higher content of Vit. C than the control sample (Kolodyazhny and Suponits, 1975). Ozone was presented to be an alternative to chlorophospham as a sprout control agent for Russet Burbank potatoes in Canada (Frungo, et al. 1967).
Carrots, inoculated with pathogenic fungi, Botrytis cinerea Pers. and Sclerotinia sclerotiorum de Bary, were exposed to a gas mixture containing 0 to 60 mg/L ozone at a flow rate of 0.5 L/min for 8 h daily for 28 days (Liew and Prange, 1994). Residual ozone concentration increased with increase in ozone supply concentration. A 50% reduction of daily growth rates for both fungi was obtained at 60 mg/L ozone. 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.

Naitoh and Shiga (1989) reported that simultaneous treatment with ozone-air mixture (0.02 - 0.2 ppm) and ozone water (0.3 to 0.5 ppm) decreased total microbial count and elongation of hypocotyls of bean sprouts (black matpe and alfalfa). Catalase and superoxide dismutase activities increased significantly with ozone treatment during germination.

Effects of treating kimchi ingredients (cabbage, hot pepper powder, garlic, ginger, green onion, and leek) with ozone gas (6 mg/L/s for 60 min) on the vitamin content, bacterial count, and sensory properties of this product were investigated (Kim, et al. 1993). Ozone treatment reduced 80 - 90% of total bacterial population in garlic and ginger and improved sensory properties of kimchi. Black pepper corn, contaminated with Salmonella spp., S. aureus, B. cereus, Penicillium spp., or Aspergillus spp., were immersed in water and sparged with ozone air (6.7 mg/L) for 10 min at a flow rate of 6 L/min (Zhao and Cranston, 1995). Ozone treatment decreased the microbial counts by 3 to 4 log.

Lewis et al. (1996) reported that ozonation (10 ppm for 10 and 50 min) did not
significantly affect beta-carotene levels of broccoli florets over eight days modified atmosphere storage, however, surface color quality was adversely affected. Zhuang et al. (1996) evaluated postharvest quality of ozone-treated broccoli florets and physiology during storage. Ozone treatment didn't deter total microbial growth after eight days storage and reduced color, ascorbic acid and chlorophyll content, ethylene formation and total soluble proteins. They concluded that short term ozone treatment did not significantly control microbial growth and could cause quality deterioration.

Winter mushrooms (Flammulina velutipes) were exposed to ozone gas at 0.03 to 0.3 ppm (Watanabe, et al. 1996). Ozone treatment during primordial formation stage resulted in a significant weight increase, however, the same ozone treatment during development stage resulted in weight decrease. This change was also related to changes in chemical composition such as carbohydrate, ash, minerals and vitamins.

Williams et al. (1996) designed a twin pass pressurized mass transfer system to improve ozone solubility in carrot wash water and obtained considerable reduction of microbial count. Ozonation was applied to pilot-scale treatment of carrot washwater (Williams, et al. 1995). At an ozone injection rate of ca. 5 g/L/h, fecal and total coliform bacteria counts decreased by > 3 log after 30 min ozonation.

There are several current patents on the preservation of fruit and vegetables by ozone technology. Cantelli (1988) developed a method based on holding the produce in a sealed container while maintaining an electrical discharge which forms ozone and nitrogen oxides, at concentration of ca. 0.05 ppm and 0.5 ppm, respectively. Karg (1990) obtained a patent for sterilization of heavily contaminated foods such as herbs, spices, fruits and
vegetables by ozone treatment. His process comprises an initial conditioning phase, treatment of gas mixture containing ozone, and elimination of residual ozone. Mitsuda et al. (1991) patented a method to sterilize food such as fish, fruit, vegetables, and beef in a processing room, packing receptacles or a refrigerator using a gas mixture which include O_3, CO_2, and/or N_2. Hurst (1993) developed a method for sanitizing food products by immersion of the product in a bath supplied with a continuous stream of ozone-containing bubbles. Rosenthal (1995) obtained a patent for sanitizing fruits with an apparatus consisting of UV and IR radiation and ozone-water.

Dry Foods. *Bacillus* and *Microoccus* are dominant bacterial genera of cereal grains, peas, beans, and spices. Counts of these microorganisms decreased 1 to 3 log by <50 mg/L ozone (Naioh et al., 1988). Naioh, et al. (1987 and 1988) studied the effects of ozone concentration (0.5 - 50 mg/L), exposure time (1 to 6 h) and temperature (5 to 50°C) on several cereal grains, cereal grain powders, peas, beans, and whole spices. With few exceptions, longer exposure time and lower temperature resulted in a higher microbiocidal activity in these dry foods. The authors found that oxidation of lipids in these commodities rarely occurred at lower than 5 ppm but was considerable at higher concentrations. Naioh et al. (1989) reported the treatment of wheat flour with 0.5 to 50 ppm ozone for 6 hours. This treatment inhibited microbial growth in namamen product and increased storage life 2 to 5 folds. During the storage time, thiamin content decreased by 4 to 17%, but sensory quality of namamen didn’t change. In a microbial decontamination study of spices by ozone, many samples showed only a slight (< 1 log) microbial
inactivation with 30 to 145 mg/L ozone residual but white pepper showed a 4.4 log reduction (Zagon et al., 1992). Oxidation also resulted in decreased essential oil content and had a negative effect on the sensory quality of some spices.

Ozone was applied in-heating peanut meal to destroy aflatoxins or greatly reduce their levels (Dollecar et al., 1968). Weight gains for ducklings and rats receiving treated meals were essentially comparable to control animals; however, treated meals had reduced protein efficiency ratios. Rayner et al. (1971) reported that ozone reduced aflatoxin in cottonseed meal and peanut meal. Contaminated cottonseed and peanut meal were hydrated and brought into contact with ozone at 75 to 100 °C to achieve substantial lowering of the aflatoxin content. With 15 mg/L for 30 min, ozone effectively decreased Aspergillus flavus and its aflatoxin from dried soup (Paulin et al., 1984). The destruction and detoxification of aflatoxin B1, G1, B2, and G2 (50 μg/mL in 4% dimethyl sulfoxide) with ozone were confirmed by Maeba et al. (1988). Aflatoxins B1 and G1 were degraded with 1.1 mg/L ozone within 5 min; however, B2 and G2 required 34.3 mg/L ozone and 50 to 60 min treatment time for comparable degradation. Chatterjee and Mukherjee (1993) studied the impact of ozone on the immunity-impairing activity of aflatoxin B1. Phagocytosis-suppressing activity of AFB1 was destroyed with gaseous ozone treatment (1.2 mg/L) for 6 min at a flow rate of 40 m3/min.

There were no significant differences in protein, starch, and glucose contents between ozone-treated and air-treated wheat grown in open-top chambers (Scotti et al., 1994). Ilsen (1989) found that in manufacturing whole milk powder, TBA value and organoleptic quality decreased with increasing concentration of ozone up to 200 ppb in the
drying air; the effect of ozone on flavor being most pronounced for freshly manufactured powder. McCabe (1985) obtained a patent to describe continuous process apparatus and method for treating foods, such as potato chips, dehydrated onions and other dehydrated vegetables, with ozone gas.

Ozone was also used to treat garlic during long-term cold storage (Galdun, et al. 1984). Ozone increased yield of standard garlic by 3.7% and reduced Penicillium mold damage. They determined that optimal conditions were ozone treatment for 6 h once every 4 days using ozone at 10 to 12 μg/L air in autumn, and once every 7 days using ozone at 5 to 6 μg/L air in winter. A flow reactor was used to study the feasibility of using ozone to oxidize odors produced during dehydration of onions and garlic (McGowan et al. 1979). Ozone treatment (5 - 20 ppm for 30 seconds) could destroy 60 to 90% of the individual gaseous components from onions and garlic oils.

Ground black pepper samples of various moisture levels were sparged with ozone air (6.7 mg/L) for up to 6 h, resulting in 3 to 6 log reduction in microbial count (Zhao and Cranston, 1995). Higher moisture content led to a greater reduction in the microbial load. Ozone treatment of ground black pepper resulted in the oxidation of certain volatile oil constituents, while the treatment had no significant effect on volatile oil constituents of whole peppercorns.

Cheese. Ozone concentration of 0.1 and 10 μg/L in the atmosphere of cheese ripening room inactivated 80 to 90 and 99%, respectively, of mold spores without affecting the organoleptic qualities of the cheeses (Skiler et al. 1978). Batches of Rossiliski,
Poshekonskii, Kostroma and Swiss-type cheeses were stored at 2 to 4 °C and 85 to 90 % RH with ozonization of air (Gabriel's ants et al. 1980). The researchers found that periodic ozonization for at least 4 h at 2 to 3 day intervals with 5 to 7 μg/L ozone in air prevented the growth of molds on the cheeses and packaging materials for 4 months without adversely affecting chemical and sensory properties of the cheese. Control cheese exhibited mold growth as early as at one month. Horvath et al. (1985) noted that storage life of cheese was increased to 11 wks by the application of small ozone concentration (0.02 mg/L) during the ripening period. Spores on the surface of cheese were destroyed by this treatment. Other experiments conducted on cheddar cheese also indicated that the oxidizing action of ozone removes odors otherwise present in storage rooms. Shiler et al. (1983) described a method of ozonation for ripening and storing cheese to inactive contaminating microflora but to avoid damage to cheese packaging materials and to improve hygiene. For optimum results, ozonization was carried out for 1 to 3 h/day at an ozone concentration of 0.08 to 0.1 μg/L with intervals of 2 to 12 h, and every 10 to 30 days the chambers were treated with ozone in a concentration of 8 to 12 μg/L for 2 to 4 h. Volodin and Shiler (1978) tested the applicability of different plastics films for cheese packaging. They reported that thin films as VIM-K, Novallen-K and Saran (12 μm) were suitable for surface sterilization treatment of packaged cheese with ozone because of their ozone permeability; however, thick films such as VIM-D (260 - 470 μm), Novallen-D (300 - 480 μm), Saran (37 μm), Hostaphan (90 μm), and KOD 115 (76 μm) were not permeable to ozone.

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Fish. Haraguchi et al. (1969) studied the preserving effect of ozone on fresh jack mackerel (Trachurus trachurus) and shimaaji (Caranx mortensi). Treatment of the skin of the gutted fish with 3% NaCl solution containing 0.6 ppm of ozone for 30 to 60 min decreased the viable bacterial count by 2 to 3 log. The storage life of the fish increased 20 to 60% when the ozone treatment was applied every 2 days. Chen et al. (1987) studied ozone for in-plant sterilization of frozen fishery products. They found that ozone was effective in distilled water and 3% NaCl solution for the inactivation of microorganisms such as Vibrio cholera, E. coli, S. typhimurium, V. parahaemolyticus, and S. aureus. However, ozone was not effective for sterilizing shrimp meat and the treatment reduced E. coli only 98.5%. Coudrains and Starck (1988) applied ozone at a concentration of 10 to 15 mg/L air for 4 to 6 min to remove odor and color from fish flesh. Dondo et al., (1992) reported that ozone decreased surface contaminants of fish during several days of refrigerated storage. Ozone treatment improved the sensory quality of fish by decreasing the formation of trimethylamine. A beneficial decoloration effect of horse mackerel (Trachurus japonicus) mince resulted from washing with ozonized water for 10 to 20 min (Chen, et al. 1997). However, a marked decrease in pH and an undesirable gel strength of mince, as well as oxidation of the fish oil, occurred during this ozone treatment. Ozone promoted detachment of the surface slime of the redfish aboard fishing vessels and ozonation during transport reduced bacterial count and improved shelf life by about 1.5 days (Koetter, 1997). Simulation trials in the laboratory indicated that bacterial counts were higher on fish held in ozonated water than on control fish. The author attributed this difference to the lower freshness of redfish used in the laboratory. Therefore, it is
recommended that fish should be treated with ozone when it is fresh. Ozone was tested to improve the washing process which is applied during the manufacture of dark-fleshed fish surimi (Chen and Lao, 1997). The investigators found that ozone washing treatment minimized the washing time and improved color, however, undesirable gel strength was observed as well as a decrease in the pH of the minces.

**Water and fluid food.** Due to the environmental disposal problems associated with chlorinated sanitizers, ozone is being explored for use in the dairy and fluid food industries. Sander (1985) developed an ozone treatment method for fruit juices and liquid dairy products to avoid possible quality deterioration resulting from conventional ozone treatments. Rojek et al. (1995) attempted to use pressurized ozone to decrease the microbial population of skim milk. In this study, ozone gas concentration was 5 to 35 mg/L, and treatment time was 5 to 25 min. Their results showed that pressurized ozone was effective in decreasing psychrotrophic counts by 2.4 log when ozone passed through skim milk. Treatment of whey and apple juice produced favorable microbial reduction. Greene et al. (1993) proved ozone effectiveness against biofilms of milk spoilage bacteria, such as *P. fluorescens* and *Aeromonas faecalis*, on stainless steel plates. Greater than 99% of population was eliminated by ozone treatment at 0.5 ppm for 10 min. Hurst (1995) developed a method for disinfecting food products using ozone-containing bubbles. When the food product is immersed in the bath containing the bubbles, the ozone in bubbles comes in contact with food surface and thus sanitized the product.

Franz and Gagnon (1971) investigated an ozone treatment to sterilize contaminated
spring water for use in the food industry. They found that coliforms and spore-forming bacteria were inactivated with eight min treatment at 0.1 to 0.2 mg/L and 1.6 to 3.2 mg/L ozone, respectively. However, in an industrial installation, only 80% sterilization was achieved with 14 min and ozone concentration of 1.12 to 2.18 mg/L. Ozone consumption was increased by high contents of suspended matter and by raising pH. They also reported that preliminary floculation decreased ozone consumption and produced completely germ-free water. Possible applications of ozone in the brewery were suggested (Tenney, 1973). These include yeast washing, selective removal of bacterial contaminants and final rinses of bottles, cans, fillers, pipelines and tanks.

Ozone was used for ageing a fermented product, such as a distilled liquor (Leu et al. 1992). Ozone is considered with other treatment as a means of ensuring water quality in the beverage industry (Fritsch, 1994). Hargesheimer and Watson (1996) reported that ozone altered the fishy odor, associated with some phytoplankton blooms in source of drinking water, to an undesirable ‘plastic-like’ odor. They suggested a combination of granulated activated carbon with ozonation for removal of particulates, color, taste and odor compounds. The water for ice manufacture may also be sterilized with ozone (Anon. 1995).

Process water and Effluents. Woerner et al. (1970) examined direct ozonization to disinfect protein-containing fluid synthetic media, household effluent and slaughter house effluent. They found that 5 to 10 mg/L gaseous ozone was adequate to eliminate bacteria according to the degree of contamination. Salmonellae were eliminated after a contact time
of seven min and anthrax spores after 30 min. While describing possible methods for sterilization of slaughterhouse effluents, Boehm (1989) suggested ozone treatment as the best chemical method. Hurst (1991) patented a method by which ozone is bubbled through the food process water to remove fat, bacteria, solids and other impurities then to reuse this water. Post-process spoilage of canned food was decreased by using ozone treated water for cooling cans (Ito and Seeger, 1980). Loorits et al. (1975) explored a possibility of using ozone for oxidizing major milk components. Ozone reduced the fat content in condensates (80 - 230 mg/L) by 96 to 98 % and completely eliminated turbidity. They concluded that ozone treatment could be applied to the purification of lightly polluted dairy effluent for subsequent reuse in water supply systems. The chemical oxidation of olive mill effluents by ozone was developed to reduce chemical oxygen demand, aromatic content and phenolic content (Benitez, et al. 1996).

By-products. Egg shells were broken into small pieces and subjected to the action of ozone. After the shell became fine powder through freeze-drying process, it was again subjected to ozone to make it free of bacteria (Monceaux, 1969). Ozone was used to destroy the porphyrine structure in swine hemoglobin and to prepare decolorized protein products (Chang et al. 1996a). The same authors (1996b) also reported that amino acid analysis and sulphydryl group determination demonstrated that cysteines and disulphide bonds were completely destroyed during the decolorization process.
Processing plant. A factory that manufactures plastic films was treated with ozone at 0.02 to 0.16 ppm for 10 h per day and 1 to 1.5 yrs (Naitoh, 1993). Aerial contaminants such as Bacillus and Micrococcus spp. in the plastic film processes was reduced. Chun et al. (1993) developed an UV air cleaner for the sterilization and deodorization of the air in refrigerators. The authors reported that ozone production reached 0.082 ppm in the holding section at 25°C and 0.06 ppm at 3°C. Disinfection of air is an important part of clean room technology in the food industry. Holah et al. (1995) evaluated different air disinfection system and found that ozone was effective and reproducible in its effect on airborne microorganisms. Ozone also can be applied for preventing secondary contamination during bread manufacturing (Staszewska, 1994). The bactericidal action of activated oxygen (O, O₂, and O) destroys or reduces organisms on food preparation surfaces, and inhibits developments of cold tolerant bacteria and pseudomonads on foods (Anon. 1994 b). Decupper (1992) obtained a patent to use ozone and a UV sterilization unit for cold storage for foods.

Greene et al. (1994) tested the resistance of standard-molded, one piece 0-ring food processing plant gaskets (36.1 mm) made of seven different substances [Buna N, white Buna N, ethylene propylene diene monomer (EPDM), polyethylene, silicone rubber, Teflon (PTFE) and steam-resistant Viton] against a chlorine sanitizer and ozonated water (0.4 - 0.5 ppm). Ozone treatment affected the tensile strength of EPDM and Viton, but not significantly more than chlorine treatment. The elasticity of ozone-treated PTFE gasket was significantly different from chlorine-treated ones.
Miscellaneous applications. Karg (1986) developed an ozone treatment process using gaseous or supercritical ozone for non-food products, plants, herbs or spices. Green tea was converted into black tea by heating an aqueous solution of green tea solids at pH greater than 6.0 in the presence of ozone (Graham et al. 1969). Ozone can be introduced to prevent bean expansion in steeping raw coffee beans and also used to treat roasted beans (Weiss and Weiss, 1971). Ozone treated beans do not contain H₂S and have a bright and smooth surface. A process for preservation and/or sterilization of foods and tobacco products is also based on fumigation with ozone (Bundschuh and Rilling, 1996). Ozone sterilization of food packaging materials can result in oxidation of antioxidants used in such packaging (Steiner, et al. 1994). They suggested that migration of ozonated compounds into foods may affect sensory properties.

While Rice et al. (1982) described the applications of ozone for perishable foods in storage, they pointed out that problems of surface oxidation of foods reported from the earlier studies were due to the excessive use of ozone at high concentrations. In addition, they stressed that ozone is not universally beneficial, and in some cases may promote oxidative spoilage. Potential problems exist in the reactions of ozone with ethylene to produce ethylene oxide and chlorohydrins in the presence of chloride ions, and in the formation of bromates, which are long-lived oxidants, when ozone is used to sterilize seawater for production of ozone-sterilized ice for fish preservation. Both of these side products may be toxic in some circumstances.
Conclusion

Previous research indicates that ozone can be used as a safe and effective antimicrobial agent in many food applications. Lower concentrations of ozone and shorter contact times are sufficient, compared to chlorine and other agents, in controlling or reducing microbial population. Ozone is also more effective than other disinfectants against resistant organisms such as amoebic cysts and viruses. Exposure to ozone during processing or storage extends the shelf life of certain products such as fruits and vegetables while maintaining their organoleptic properties. Ozone does not produce significant toxic residues in the environment after treatment.

More studies are required, however, to better understand kinetics and mechanism of inactivation of microorganisms by ozone, and feasibility and limitations of using ozone in food processing. Future studies may also address the synergistic effect of ozone and other processing conditions or preservation factors on the safety of foods. With acquired knowledge and experience, operating specifications and regulations can be developed to use ozone at the most efficient and safe level.
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<th>Bacterium</th>
<th>Inactivation (Log$_{10}$)</th>
<th>Treatment time (min)</th>
<th>Concentration (mg/L)</th>
<th>pH</th>
<th>Temp. (°C)</th>
<th>Medium</th>
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<td>0.12</td>
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<td>2.29</td>
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<td>0.23-0.26</td>
<td>7</td>
<td>24</td>
<td>O$_3$, demand-free water</td>
<td>Continuous flow</td>
<td>Farooq &amp; Akhlaque (1983)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3.0</td>
<td>19</td>
<td>Initial 2.2, residual 0.06</td>
<td>7.5</td>
<td>16</td>
<td>Raw wastewater</td>
<td>Continuous flow</td>
<td>Ioret et al. (1982)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>2.0</td>
<td>0.33</td>
<td>0.065</td>
<td>7</td>
<td>1</td>
<td>Water</td>
<td>Batch</td>
<td>Peters and Ingel (1959)</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>&gt;4.5</td>
<td>20</td>
<td>0.32</td>
<td>7</td>
<td>24</td>
<td>Distilled water</td>
<td>Batch</td>
<td>Edstein et al. (1982)</td>
</tr>
<tr>
<td><em>M. fumatum</em></td>
<td>1.0</td>
<td>1.67</td>
<td>0.23-0.26</td>
<td>7</td>
<td>24</td>
<td>O$_3$, demand-free water</td>
<td>Continuous flow</td>
<td>Farooq &amp; Akhlaque (1983)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>&gt;2.0</td>
<td>0.25</td>
<td></td>
<td>7</td>
<td>24</td>
<td>O$_3$, demand-free water</td>
<td>Continuous flow</td>
<td>Burleson et al. (1975)</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>1.6</td>
<td>0.25</td>
<td>8% (w/w)</td>
<td>25</td>
<td></td>
<td>Broiler carcass</td>
<td>Ozone gas</td>
<td>Ramirez et al. (1994)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>4.3</td>
<td>1.67</td>
<td>0.23-0.26</td>
<td>7</td>
<td>24</td>
<td>O$_3$, demand-free water</td>
<td>Continuous flow</td>
<td>Farooq &amp; Akhlaque (1983)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&gt;2.0</td>
<td>0.25</td>
<td></td>
<td>7</td>
<td>25</td>
<td>Phosphate buffer</td>
<td>Batch (bubbling)</td>
<td>Burleson et al. (1975)</td>
</tr>
</tbody>
</table>

Table 1.1. Inactivation of bacteria by ozone.
<table>
<thead>
<tr>
<th>Fungus</th>
<th>Inactivation (Log)</th>
<th>Treatment time (min)</th>
<th>Concentration (mg/L)</th>
<th>pH</th>
<th>Temp. (°C)</th>
<th>Medium</th>
<th>Reactor type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida parapsilosis</td>
<td>2.7</td>
<td>1.67</td>
<td>0.23 - 0.26</td>
<td>7</td>
<td>34</td>
<td>O₂ demand-free water</td>
<td>Continuous flow</td>
<td>Panooq &amp; Akhlaque (1983)</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>2.0</td>
<td>0.30, 0.08</td>
<td>0.02, 1</td>
<td>7.2</td>
<td>20</td>
<td>O₂ demand-free water</td>
<td>Continuous flow</td>
<td>Kawamura et al. (1986)</td>
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Table 1.2. Inactivation of fungi by ozone.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Inactivation (Log)</th>
<th>Treatment time (min)</th>
<th>Concentration (mg/L)</th>
<th>pH</th>
<th>Temp. (°C)</th>
<th>Medium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriophage f2 effluent</td>
<td>0.7</td>
<td>10</td>
<td>0.1</td>
<td>7.2</td>
<td>20</td>
<td>Activated sludge effluent</td>
<td>Harakeh &amp; Butler (1985)</td>
</tr>
<tr>
<td>Bacteriophage f2</td>
<td>&gt;4.3</td>
<td>0.16</td>
<td>0.4</td>
<td>7</td>
<td>20</td>
<td>Water</td>
<td>Boyce et al. (1981)</td>
</tr>
<tr>
<td>Coxsackie virus B5</td>
<td>4.0</td>
<td>2.5</td>
<td>0.4</td>
<td>7.2</td>
<td>20</td>
<td>Sludge effluent</td>
<td>Harakeh &amp; Butler (1985)</td>
</tr>
<tr>
<td>Coxsackie virus A9</td>
<td>&gt;1.7</td>
<td>0.16</td>
<td>0.035</td>
<td>7</td>
<td>29</td>
<td>Water</td>
<td>Boyce et al. (1981)</td>
</tr>
<tr>
<td>Enteric virus</td>
<td>&gt;1.7</td>
<td>29</td>
<td>Initial 4.1, residual 0.02</td>
<td>7.8</td>
<td>18</td>
<td>Raw wastewater</td>
<td>Iovet et al. (1983)</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>2.7</td>
<td>0.02</td>
<td>0.25</td>
<td>7.2</td>
<td>20</td>
<td>Phosphate buffer</td>
<td>Herbst et al. (1987)</td>
</tr>
<tr>
<td>Human rotavirus</td>
<td>0.7</td>
<td>10</td>
<td>0.3</td>
<td>7</td>
<td>20</td>
<td>Sludge effluent</td>
<td>Harakeh &amp; Butler (1985)</td>
</tr>
<tr>
<td>Polyovirus type 1 (Algonquin)</td>
<td>2.5</td>
<td>1.67</td>
<td>0.23-0.26</td>
<td>7</td>
<td>24</td>
<td>Na demand-free water</td>
<td>Parooq &amp; Akhlaque (1983)</td>
</tr>
<tr>
<td>Polyovirus type 1 (Algonquin)</td>
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<td>0.53</td>
<td>0.51</td>
<td>7.2</td>
<td>20</td>
<td>Water</td>
<td>Roy et al. (1981)</td>
</tr>
<tr>
<td>Polyovirus type 1</td>
<td>2.0</td>
<td>10</td>
<td>0.2</td>
<td>7.2</td>
<td>20</td>
<td>Activated sludge effluent</td>
<td>Harakeh &amp; Butler (1985)</td>
</tr>
<tr>
<td>Recovirus</td>
<td>&gt;2.0</td>
<td>0.25</td>
<td>7</td>
<td>25</td>
<td></td>
<td>Phosphate buffer</td>
<td>Bulson et al. (1975)</td>
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Table 1.3. Inactivation of viruses by ozone
<table>
<thead>
<tr>
<th>Protoplast</th>
<th>Inactivation Treatment</th>
<th>Concentration</th>
<th>pH</th>
<th>Temp. °C</th>
<th>Reactor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. plicatula</td>
<td>UV, 5mins</td>
<td>1</td>
<td>7</td>
<td>25</td>
<td>Batch</td>
<td>Sato et al. (1990)</td>
</tr>
<tr>
<td>Giardia lamblia</td>
<td>O3, 0.1ppm, 1hr</td>
<td>1.1</td>
<td>7</td>
<td>25</td>
<td>Batch</td>
<td>Wickramasinghe (1994)</td>
</tr>
<tr>
<td>Giardia muris</td>
<td>O3, 0.1ppm, 1hr</td>
<td>0.7</td>
<td>7</td>
<td>25</td>
<td>Batch</td>
<td>Wickramasinghe (1994)</td>
</tr>
<tr>
<td>Nogardia granulosis</td>
<td>UV, 5mins</td>
<td>2.0</td>
<td>7</td>
<td>5</td>
<td>Water</td>
<td>Wickramasinghe (1994)</td>
</tr>
<tr>
<td>Nogardia granulosis</td>
<td>UV, 5mins</td>
<td>2.0</td>
<td>7</td>
<td>5</td>
<td>Water</td>
<td>Wickramasinghe (1994)</td>
</tr>
</tbody>
</table>

Table 1.4 Inactivation of protoplasts by ozone.
Figure 1.1. Conceptual design of corona discharge ozone generator (adapted from Rosen, 1972). (A) Basic configuration, (B) Tube-type generator unit.
CHAPTER 2

INACTIVATION OF SELECTED FOODBORNE BACTERIA BY OZONE

Abstract

Ozone was tested against Pseudomonas fluorescens, Escherichia coli O157:H7, Leuconostoc mesenteroides, and Listeria monocytogenes in deionized water. Results from the batch reaction system show that E. coli O157:H7 was the most resistant and L. monocytogenes was the least resistant to ozone, compared with other tested bacteria. When data of inactivated population from the batch reactor were fitted to a dose-response plot, a two-phased linear relationship was observed with all bacteria. Bacterial inactivation increased greatly when the concentration of ozone increased from 0.15 to 1.2 ppm. Higher concentrations of ozone caused limited increase in inactivation rates.

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. Washed bacterial cells were captured on an ozone-resistant filter, which was mounted on
a fiber-glass disk of a filtration unit and ozonated water was passed through the unit. Survivor's plots of all the tested microorganisms 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 resulted in a 5-6 log decrease in count. Resistance of tested bacteria to ozone followed this descending order: E. coli O157:H7, P. fluorescens, Leu. mesenteroides, and L. monocytogenes.

Introduction

Inactivation of bacteria by ozone was largely studied in batch systems and single end-point determinations of viability were reported (Edelstein et al., 1982; Farooq et al., 1977; Fitz et al., 1988; Hunt and Marinas, 1997; Katzenelson et al., 1974). Therefore, results on relative resistance of foodborne spoilage and pathogenic bacteria to ozone were inconclusive (Boyce et al., 1981; Broadwater et al., 1973; Farooq and Akbilaque, 1983; Restaino et al., 1995). Additionally, information is lacking about inactivation of emerging foodborne pathogens (e.g., Listeria monocytogenes and Escherichia coli O157:H7) with ozone.

Inactivation of bacteria by ozone is likely to follow different kinetics depending on the species of treated microorganisms. Before ozone can be applied successfully in food processing, patterns of microbial inactivation by ozone should be elucidated. Therefore, the objectives of this investigation are to (a) measure the ability of ozone to inactivate selected foodborne microorganisms, (b) develop a method to study patterns of microbial inactivation.
by ozone, and (c) use the inactivation data to predict the inactivation of microorganisms at a broad range of ozone concentrations.

**Material and Methods**

**Preparation of inoculant**

*P. fluorescens*, *E. coli O157:H7*, *Leu. mesenteroides*, and *L. monocytogenes* were obtained from the culture collection at The Ohio State University (Columbus, OH). Inoculum of *L. monocytogenes* Scott A was prepared as described by Lou and Yousef (1996). *L. monocytogenes* was cultured and stored at -20°C in Trypticase soy broth (BBL, Cockeysville, MD) supplemented with 0.6% Bacto yeast extract (Difco Laboratories, Detroit, MI) (TSBYE) and 10% (v/v) glycerol. The stock culture was inoculated into TSBYE and the mixture was incubated at 35°C for 24 hr; 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 (4°C) centrifuge (Sorvall RC-5B Superspeed Centrifuge, DuPont Instruments, Wilmington, DE) and washed twice in 0.1 M phosphate buffer solution (pH 7) to a final concentration of 1-3 x 10⁸ CFU/mL. Inocula of the other bacteria were prepared using a similar protocol but growth media and incubation conditions were different.

*E. coli O157:H7* was subcultured twice in Trypticase soy broth (TSB) and incubated at 35°C for 24 hr. *P. fluorescens* was grown in nutrient broth (Difco) and incubated at 26°C for 24 hr. *Leu. mesenteroides* was cultured in L.acidobacilli MRS broth.
(Difco) and incubated at 26°C for 24 hr. Inoculum sizes were estimated by measuring absorbance at 600 nm (A_{600}) and calculating approximate counts from the standard curve for absorbance vs bacterial count.

**Ozone demand-free glassware and water**

All glassware were washed with a mild detergent and thoroughly rinsed with hot tap water and deionized water. They were then autoclaved and dried to remove volatile organic compounds. Deionized water was obtained from a Milli-Q system (Model OM-140, Millipore Corp.). Ozone demand-free water was prepared by ozonating deionized water (Fig. 2.1). The water was then autoclaved at 121°C for 15 min to remove residual ozone and stored in sealed ozone demand-free glass containers until needed (Korich et al., 1990). Water was buffered to pH 6, 7, and 8 with 0.01 M phosphate. Citrate-phosphate buffer (0.01 M) was used for pH 5 and 9.

**Ozone production**

Ozone (2.0 mM; ~5%, v/v; ~7%, w/w) was produced from purified, extra dry oxygen by an ozone generator (U.S. Filter/Polymetrics T-816, San Jose, CA). The amount of ozone produced by the generator and that available for the treatment were determined as indicated later.
Measurement of ozone concentration

Ozone concentration was determined by ultraviolet (UV) spectrophotometry and the indigo method (Stader and Hoigne, 1981). In case of the UV method, the concentration of ozone in aqueous solution was determined continuously by measuring UV absorption at 258 nm ($A_{258}$) in a spectrophotometer (Spectronic 1201, Milton Roy Co., Rochester, NY). A standard curve between UV absorbance and ozone concentration by the indigo method was obtained to estimate ozone concentration (Fig. 2.2). In case of the indigo method, ozone was determined by decolorization of indigo trisulfonate (Aldrich Chemical Co., Inc., Milwaukee, WI) and measurements of changes in color at 600 nm, and pH <4. The indigo molecule contains only one C=C double bond which reacts with ozone at a very high reaction rate constant. Ozone concentration was calculated using the following equation.

Ozone concentration in mg O$_3$/L = $dA * 1000/(b*V)$

where,

$dA$ = difference in absorbance between sample and blank

$b$ = path length of the cuvette in cm

$V$ = volume of the ozone water sample added in mL

$f = 0.42$

Preparation of aqueous ozone

Ozonated water was obtained by bubbling ozone ~ 5% (v/v, in oxygen carrier gas) into a round-bottom flask containing ~1000 mL sterile deionized water at 25°C (Fig. 2.1). The flow rate of ozone into the flask was controlled by a peristaltic pump (Masterflex,
Cole-Parmer, Vernon Hills, IL) delivering 0-30 mL/min. A stainless-steel sparger with 10 μm pore size (Solvent Inlet Filter, Fisher Scientific, Fair Lawn, NJ) was used for bubbling ozone in the water. The ozonated water was circulated by a peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL) with a flow rate of 8.1 mL/min through Norprene tubing (Cole-Parmer, Vernon Hills, IL) to the spectrophotometer's flowcell (0.6 mL capacity, Fisher Scientific, Fair Lawn, NJ) 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 258nm remained relatively constant.

Inactivation studies

A batch and three continuous reaction systems were set up to study the inactivation of selected microorganisms by ozone. Because of reactivity of ozone, only glass containers and tubes and Norprene tubing were used in the setup. 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 predetermined volumes of ozonated water and cell suspension in a container and neutralizing the mixture after it was held for a given time (Fig. 2.3). A reaction vessel containing 95 mL water was ozonated, at a given pH and temperature, to a steady-state concentration as determined by absorbance measurement. The vessel was inoculated with 5 mL cell suspension (in 0.05 M phosphate buffer) to attain a count of ~10^6 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 sodium thiosulfate solution (0.206 g/L; one-tenth the sample volume) in a tube to halt the reaction. Cells were counted for all samples by the standard plate counting method. In some experiments, ozonated water (9.5 mL) was mixed with 0.5 mL cell suspension and the mixture was held for 30 sec before an 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 reactions. Three continuous reactors (designated as I-III) were setup as follows.

Reactor I. Reactor I (Fig. 2.4) 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 7 sampling ports (three-way valves) at different lengths. Samples of equal size were collected from ports and mixed immediately with a sodium thiosulfate solution in a tube to neutralize residual ozone. Contact time was calculated as follows: (Resident volume between the mixing point to sampling port / Flow rate (mL/min)) x 60 sec. The calculated contact time ranged from 0.4 to 37.8 sec. Plate counting was performed for the obtained samples.
Reactor II. The reactor was designed to permit exposure of cells on a membrane filter (pore size, 0.45 μm; Diameter, 25 mm; composition, mixed cellulose ester; GA6-5, Gelman Sciences Inc., Ann Arbor, MI) to a steady stream of ozonated water (Fig. 2.5). Cell suspension (1 mL) was filtered using the membrane which was mounted on the stainless-steel support screen in a syringe-type filter holder (Gelman Sciences Inc.). Ozonated water was drawn into the filtration unit continuously for a predetermined time (15-150 sec) by a peristaltic pump at a flow rate of 8.5 mL/min. The syringe-type filter holder is made of Delrin acetyl resin, on which ozone may have moderate effect according to chemical resistance charts (Cole-Parmer). The membrane filter was transferred into a peptone dilution tube (15 mm size) using a tweezer to stop ozone reaction and the sample was prepared for plate counting.

Reactor III. This reactor is similar in principle but simpler in design than the previous reactor II (Fig. 2.6). A membrane filter (pore size, 0.45 μm; Diameter, 25 mm; composition, mixed cellulose acetate and nitrate; HA WP, Millipore, Marlborough, MA) was mounted on the fritted glass base of a glass filtration unit (Glass Microanalysis, Millipore) and the unit was assembled. The funnel functions as an ozonated water reservoir. Cell suspensions (1-2 mL, ~10⁸ cfu/mL) and subsequently ozonated water (1-10 mL) were drawn through the filter at a constant rate (22 mL/min) using a peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL). Contact time was calculated as follows: (Volume of applied ozonated water / Flow rate [mL/min]) x 60 sec. Dosage was varied by using variable volume (1-10 mL) of ozonated water or by applying equal volumes of water which contained different concentrations of ozone. The filter with the treated cells was
transferred into peptone water for ozone neutralization. Dilutions were made and plate counting was performed.

Neutralization

Sodium thiosulfate (0.1 N) neutralizer stock solution was prepared by dissolving 25 g of Na$_2$S$_2$O$_3$.5H$_2$O (Sigma Chemical Co., St. Louis, MO) in 1 L of freshly autoclaved distilled water (Rand et al., 1975). The amount of sodium thiosulfate solution was varied depending on the estimated ozone concentration in the solution being neutralized. When feasible, neutralizer was added to the reaction mixture in amounts sufficient to decrease A$_{340}$ to zero. According to preliminary data, amounts of neutralizer added to the test solution did not have an interfering absorbance at 254 nm, nor an adverse effect on treated microorganisms.

Microbiological tests

Microbiological methods employed were selected from the Bacteriological Analytical Manual (BAM, 1992) and the Compendium of Methods for the Microbiological Examination of Foods (Vanderzant and Splittstoesser, 1992). For the enumeration of *Pseudomonas*, *Escherichia*, and *Leuconostoc*, nutrient agar (NA), Trypticase soy agar (TSA), and Lactobacilli MRS (MRS) were used, respectively. Trypticase soy agar plus yeast extract (0.6%) was used for *Listeria* count.
Results

Ozone production and measurement

Bader and Hoigne (1981) used Ultraviolet (UV) spectrophotometry, at wavelength of 258 nm, to measure ozone. Ozonated water was scanned by a diode-array spectrophotometer (Hewlett-Packard model 8452A, Chicago, IL) in order to confirm the optimum wavelength for ozone measurement. UV absorption spectrum revealed a distinct peak at 258 nm (Fig. 2.7). This wavelength was used in ozone determination throughout the study.

Correlation between UV measurements and ozone concentration was determined. Water was ozonated to different ozone concentrations. _A_\textsubscript{258} was measured, and ozone concentration was determined by the iodigo method. Absorbances at 258 nm were linearly related with ozone concentrations (R\textsuperscript{2} = 0.993), thereby ozone concentration may be estimated from UV measurements using a standard curve-based conversion factor (Fig. 2.2).

Stability of aqueous ozone

Ozonation of different waters. Deionized and double distilled waters and tap water from two sources were bubbled with gaseous ozone (2.0 mM) at low flow rate (~30 mL/min). Ozone gas was more soluble in deionized and distilled waters than in tap waters (Fig. 2.8). Higher maximum ozone concentration was also obtained in the former two waters. pH values, measured before ozonation, were 5.6 and 5.9 for deionized and distilled
water, respectively, and 8.23 and 8.39 for tap waters (Table 2.1). Ozone stability decreases as pH increases (Fig. 2.9). Ozone decomposed rapidly in 0.01 M phosphate buffer when the pH was greater than 8.0. In addition to the high pH, tap waters may contain organic matter that consumes ozone. Therefore, solubility (ozone concentration in water/ozone concentration in gas) and stability of ozone may be related to pH and the presence of ozone demand materials in water.

Decomposition of ozone in different waters. Distilled, deionized, HPLC-grade, and tap waters, and phosphate buffer (0.05M, pH 7), were ozonated to 1.9-2.9 ppm and changes in residual ozone were monitored during storage at 25°C for 8 min (Fig. 2.10). Residual ozone of ozonated distilled, deionized, and HPLC-grade waters decreased linearly. Ozone decomposed at a faster rate in ozonated buffer and tap water than in other waters, but the rate of decrease in residual ozone varied during the storage. It is apparent that high pH and presence of ozone-demand materials enhance decomposition of ozone.

Decomposition was monitored at variable initial ozone concentration (Fig. 2.11). Rate of decomposition of ozone in deionized water was greater at higher initial ozone concentration. When initial concentration was 1.8 ppm, decomposition of ozone in 30 sec at 25°C was less than 3 %. Double distilled water gave similar results as deionized water. Therefore, double distilled and deionized waters are suitable for the preparation of low ozone-demand media.
Inactivation studies

Batch reaction. Ozone at 0.28 ppm, initially, inactivated 0.9 log of _P. fluorescens_ in 30 seconds, whereas 1.21 ppm decreased the population by 4.5 log (Fig. 2.12). When _E. coli_ O157:H7 was treated with 0.25 and 1.04 ppm ozone, the count decreased 1.3 and 3.8 log, respectively, in 30 seconds (Fig. 2.13). Sensitivities of _E. coli_ O157:H7 and _P. fluorescens_ to ozone were somewhat similar. Concentration-time (C*t; ppm x min) value of _P. fluorescens_ is 0.14 for 0.85 log decrease and 0.61 for 4.8 log decrease, while C*t for _E. coli_ O157:H7 is 0.13 for 1.5 log decrease and 0.52 for 3.8 log decrease.

Counts of _Leu. mesenteroides_ decreased by 1.3 and 3.3 logs when initial ozone concentrations were 0.31 and 1.49 ppm, respectively (Fig. 2.14). Ozone at ~4 ppm killed ~7 log of _Leu. mesenteroides_. Ozone at 0.36 and 0.83 ppm, initially, inactivated ~4 and ~5 log of _L. monocytogenes_ population (Fig. 2.15).

Microorganisms tested in this study showed similar inactivation pattern. Most inactivation occurred during the first 15 or 30 seconds of the treatment and counts remained unchanged when the mixture was held for up to 6 min. Correlation between amounts of ozone remaining in the reaction mixture (residual ozone determined by indigo method) and degree of inactivation was studied.

A cell suspension of _Leu. mesenteroides_ (~10^7 cfu/mL) was mixed with ozonated water to obtain 0.31 to 2.1 ppm ozone, initially. The mixture was sampled to determine counts of survivors and the residual ozone (Fig. 2.16). When the initial ozone concentrations were 0.31, 1.14, and 2.1 ppm, 0, 0.31, and 0.60 ppm residual ozone concentrations were detected after 15 seconds, respectively. Bacterial counts after 30
seconds of exposure were 2.15, <1, and <1 log cfu/mL, respectively. Therefore, inactivation of bacteria by ozone is a rapid process. Estimated ozone demand of 10^6 \textit{Leu. mesenteroides} cfu/mL is 0.83 ppm. Therefore, ~10^{23} molecule of ozone were used to inactivate each cell.

Inactivation of \textit{Leu. mesenteroides} by 1.26 ppm ozone was investigated when initial count varied (Fig. 2.17). When initial inoculum sizes were 6.8, 7.1, 7.4, and 7.7 log cfu/mL, the decrease in ozone concentration was 0.8, 3.72, 1.07, and 1.26, and thus, the percent ozone decrease was 61.8, 58.2, 82.9, and 100, respectively. A large decrease in residual was observed initially, and the rate of decreased diminished when the mixture was held for up to 3 min (Fig. 2.17). A similar trend of inactivation patterns was observed (i.e., rapid decrease in count initially and minimal changes later). Therefore, effectiveness of ozone varied considerably with inoculum sizes. Inactivation by 1.26 ppm ozone was less than one log for the largest inoculum (7.68 log cfu/mL), but it was >6 log when an inoculum half the size of the former (7.36 log cfu/mL) was used. Therefore, ratio between amounts of treated cells and added ozone should be considered carefully for maximum effectiveness of ozone. To study inactivation at 0 to 1.3 ppm ozone in the batch reactor system, it is necessary to use large inoculum otherwise treated microorganisms becomes undetectable early in the treatment.

In the batch reaction system, microorganisms are inactivated rapidly (<30 sec) and thus determination of inactivation kinetics is technically difficult. 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 (Fig. 2.18). Data were fitted by 2 segments that were linear at two ranges of ozone concentrations. *P. fluorescens* PI values changed considerably with ozone concentration up to about 1 ppm. But these values increased moderately at concentrations >1 ppm. Similarly, data on *E. coli* O157:H7 were fitted to two linear segments of dose-response plot, these segments intersect at ~1.2 ppm initial ozone concentration (Fig. 2.19). At 1 ppm ozone, PI-values for *P. fluorescens* and *E. coli* O157:H7 were 4.6 and 2.7 log, respectively.

Data on *L. mesenteroides* also gave a dose-response plot with two linear segments (Fig. 2.20). However, unlike *Pseudomonas* and *E. coli*, *Leu. mesenteroides* gave lines that intersect at 2.1 ppm initial ozone concentration. Inactivation of *L. monocytogenes* by ozone also resulted in a linear dose-response line having two segments that intersect at 0.72 ppm initial ozone concentration (Fig. 2.21).

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 7.46 log for *P. fluorescens*, *E. coli* O157:H7, *L. mesenteroides*, and *L. monocytogenes*, respectively. *E. coli* O157:H7 was more resistant than other microorganisms, while *L. monocytogenes* was the least resistant against ozone inactivation. Increase in PI values with increase in initial ozone concentration (i.e., slope of the first segment of the dose-response plots) was greatest in case of *Listeria monocytogenes*. In conclusion, ozone concentration and cell inactivation are linearly-related over two ranges of ozone concentrations.

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Continuous reactions.

Reactor I. Unlike the batch system, this reactor permitted measuring bacterial inactivation after short periods (0.4 to 53 sec) of exposure to ozone (Fig. 2.4). When *P. fluorescens* was exposed to 0.44-0.71 ppm ozone, most of the decrease in count occurred during the early stage of the treatment (Fig. 2.22). With 0.44 ppm of ozone, ~1.5 log maximum reduction was obtained. A concentration of 0.7 ppm was required to inactivate ~2 log *P. fluorescens*. Similar inactivation patterns were noted for *E. coli* O157:H7 (Fig. 2.23). Ozone at 0.4 ppm decreased the population of *E. coli* by 2 log cfu/mL.

*Leu. mesenteroides* also showed a similar pattern of inactivation as the previous two microorganisms (Fig. 2.24). Its resistance to ozone was similar to *P. fluorescens*; the count decreased by ~2 and 3 log with 0.5 and 0.6 ppm ozone, respectively.

Counts of *L. monocytogenes* decreased gradually as contact time increased (Fig. 2.25). This inactivation pattern is more distinctive than that seen in other bacteria. Resistance of *Listeria* against ozone was the least among the tested microorganisms; ~0.4 ppm ozone inactivated ~7 log.

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. Reactor II permits continuous exposure of cells on a membrane filter, to a constant ozone concentration during the treatment (Fig. 2.5). The pattern of inactivation, however, is similar to that observed in the previous two reactors. Moreover, results were not consistent (Fig. 2.26). Uneven exposure of cells to ozone and possible ozone demand by apparatus components may have caused these inconsistencies.

Reactor III. Inactivation patterns were different with this reactor than with the previously-tested systems; survivors and log-log dose-response plots were linear over a broader range of contact times and ozone concentrations, respectively. The inactivation plots of *P. fluorescens* by ozone in the reactor III are shown in Fig. 2.27. Ozone at 0.6 and 1.4 ppm inactivated 2 and 3 log in 20 and 15 sec, respectively. Inactivation of *E. coli* O157:H7 by ozone also followed a concave downward curve at all ozone concentration tested (Fig. 2.28). With 0.4 and 0.9 ppm of ozone, -2 and -3 log reductions were obtained in 38 seconds, respectively.

Survivors plots of *Lec. mesenteroides* were also concave downward like the previous two microorganisms; however, the inactivation was quite linear for longer contact time (Fig. 2.29). With 0.75 and 1 ppm of ozone, *Leuconostoc* population decreased by -2 and -3 log cfu/mL in 38 seconds, respectively. *L. monocytogenes* also followed similar inactivation patterns as other microorganisms (Fig. 2.30). Two and three log cfu/mL were destroyed by 0.5 and 1.0 ppm of ozone in 37 seconds, respectively.

D-values were calculated from initial slopes of survivors plots, i.e., during the first 5.4 to 8.5 seconds of exposure to ozone. D-values at different ozone concentrations were plotted against ozone dosage to construct log-log dose-response plots. Inactivation of *P.
fluorescens by ozone gave a scattered dose-response plot \( R^2 = 0.64 \) but the relationship between D-value and ozone dosage was clearly linear (Fig. 2.31). The log-log dose-response model also fits inactivation data on E. coli O157:H7 (Fig. 2.32). The correlation between log D-value and log ozone concentration was 0.92. The relationship between log D-value and log ozone concentration for the inactivation of L. mesenteroides and L. monocytogenes also was linear (Fig. 2.33 and 2.34). For the comparison of ozone resistance, D-values at 1 ppm ozone treatment for the tested microorganisms were calculated. They are 4.5 sec for P. fluorescens, 6.0 sec for E. coli O157:H7, 4.1 sec for L. mesenteroides, and 3.2 sec 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 continuous reactor III. Therefore, it may still be true that different dose-response models exist depending on the ozone concentration and the ozone-demand substances present in the reaction.

Discussion

Ozone measurements

Ozonated water absorbs UV energy with a maximum at 258 nm (Fig. 2.7). This absorption occurs, however, in a UV region where other solutes interfere. Humic materials in natural water often cause a significant background absorption which varies with
ozonation (Bader and Hoigne, 1981). Moreover, due to the low molar absorptivity of ozone (2900 M<sup>-1</sup> cm<sup>-1</sup>), the sensitivity of methods based on UV measurements are relatively low. This direct spectrophotometric method, however, is useful in calibration of ozone concentrations whenever the limitations of sensitivity and background absorptions are not relevant (Bader and Hoigne, 1981).

Exposure of ozonated water to UV energy reduces ozone to molecular oxygen. Additionally, some auto-decomposition of ozone inside tubing from the reactor flask to the spectrophotometer's flow cell is expected. Therefore, UV measurements in our preliminary studies may have led to an underestimation of ozone concentrations. To overcome this drawback, a relationship between UV measurement and ozone concentration (measured by the indigo method) was obtained, under the conditions prevailed in this study. This relationship was used to estimate ozone during the remainder of the investigation (Fig. 2.2). White (1986) emphasized that for an accurate and reliable installation of a continuous oxidant analyzer, the shortest and smallest line possible should be used in order to get the sample to the analyzer cell as quickly as possible (<1 min). These and other precautions were taken during this study.

Ozone production

Before completing the inactivation studies, it was necessary to determine the solubility and decomposition rates of ozone in different waters and to choose the appropriate medium for the inactivation studies. Malle-ialle (1982) emphasized the significance of following phenomena in relation to ozonation and auto-decomposition of
ozone in water: a) transfer of ozone from the gas phase to the liquid phase; b) relation between the partial pressure of the gaseous ozone and its solubility in an aqueous solution; c) mass transfer of the ozone dissolved to trace impurities present in the water; d) kinetics of the auto-decomposition of ozone in solution; and e) kinetics of the oxidation by ozone of the impurities in the water.

**Solubility of ozone.** Maximum concentration obtained from bubbling ozone (2.0 mM) in water at 29.4 mL/min for 16 min were 6.3 (0.13), 9.4 (0.2), 15.7 (0.33), and 17.5 (0.36) mg/l (mM) for tap 1, tap 2, deionized, and distilled water, respectively (Fig. 2.8). Solubility ratio was 0.06, 0.09, 0.15, and 0.16, respectively. These solubilities are smaller than those reported by other researchers. Watson (1943) reported that the solubility ratio for ozone was 0.26 at 20°C. Meddows-Taylor (1947) determined a solubility ratio of about 0.4 at 20°C. According to data provided by Horvath et al. (1985), the solubility ratio of ozone in water is 0.07-1.85. The results reported in previous studies vary appreciably, depending on reactor design, flow rate and analytical method used.

The maximum ozone level attained in this study was ~30 mg/L (ppm). Bader and Hoigne (1986) noted that stock solutions of aqueous ozone, which were prepared by continuously bubbling ozone gas (ca. 4%) through a gas-washing bottle into distilled water at 2°C, was in a steady-state at 40 mg ozone/L. The authors measured ozone concentration by the indigo method. Finch et al. (1987) bubbled ozone gas (4.8%, w/w) in 400 mL deionized water contained in a 500-mL gas absorption flask for a minimum of 0.7 h at 20°C. The resulting stock solution contained 20-23 mg ozone/L.

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Ozone solubility in a liquid is governed by Henry's Law, i.e., the weight of gas that dissolves in a given volume of a liquid, at constant temperature, is directly proportional to the pressure the gas exerts above the liquid. However, White (1979) suggested that it is necessary to achieve the maximum contactor efficiency (TE), because of the difficulty of maintaining high partial pressures of ozone above the process liquid such as potable water or wastewater. Solubility of ozone also increases as temperature decreases.

**Mass transfer of ozone.** In our study, a sparger with 10\(\mu\)m pore size was used to decrease the bubble diameter of ozone gas and to increase the transfer rate of gaseous ozone into water phase. In addition, stirring was done during ozonation to ensure sufficient turbulence.

The mass transfer of ozone occurs via diffusion through the air-water interface. Favorable conditions for ozone mass transfer include (a) high concentration of ozone in the carrier gas, (b) high pressure, and (c) high transfer area. (Gemella, 1972). Decreasing the diameter of ozone bubble increases the total area of exchange and the contact time between water and gaseous ozone. Harris (1972) emphasized the importance of obtaining the finest possible division of gas bubbles during application of ozone in water. Meddows-Taylor (1947) defined “contact value” as “the product of total area times the time required to rise unit distance”. He emphasized decreasing ozone bubble size to increase contact value and introducing turbulence into the water in a contact chamber. Bubbles with a diameter of 0.1 cm have ~32 times more contact value than those with a diameter of 1.0 cm (Meddows-Taylor, 1947).
Presence of organic matter determines, to a great extent, the quantity of ozone entering the water (O'Donovan, 1965). Depending on water quality (temperature, pH, salt content, and presence of pollutants), different dosages of ozone may have to be applied to attain a given ozone residual. It is obvious that the solubility of ozone is substantially influenced by the presence of impurities in the water. Presence of impurities and high pH may have caused the poor solubility of ozone in tap waters (Fig. 2.8 and Table 2.1).

Ozone stability. In this study, ozone was stable at pH values <8.0 (Fig. 2.9) decomposed faster in tap waters having pH ~8.3 (Fig. 2.10). It is rare to find municipal water supplies that are not chlorinated (Dyckiola, 1977). An alkaline pH results when preparing a chlorine solution using sodium hypochlorite. Presence of traces of oxides of nitrogen, chlorine, and phosphorous pentoxide considerably accelerates the degradation of ozone (Meddows-Taylor, 1947). The rate of decomposition of ozone in aqueous solutions may also vary with the degree of turbulence (Shechter, 1973), temperature (Sease, 1976), pH, ozone concentration and presence of organic matter. The stability of ozone in water is greatly influenced by the presence of contaminants, particularly metal ions (Horvath, et al., 1985). Therefore, deionized water of pH ~5.6 was selected as the experimental medium for inactivation study.

Kolle (1968) showed that ozonation of a 20 mg/L chlorine (hypochlorite) solution lowers the pH and causes formation of chlorine derivatives such as chlorate and perchlorate ions. However, Buydem and Fransoolet (1971) did not notice any interaction of ozone and chlorine. The germicidal efficiency of ozone does not seem to be affected significantly at pH 6-8.5 (White, 1986).
Farooq et al. (1977) investigated pH effect on ozone decomposition and inactivation rate for *M. fortuitum* and *Candida parapsilosis*. In ozone treatment of *M. fortuitum*, they found that the high degree of survival at pH 10.1 corresponds to a low ozone residual, as compared with decreased survival at pH 5.7 with a high ozone residual. They concluded that the inactivation rate is related more to the ozone residual than to the pH. On the other hand, pH influences the rate of ozone decomposition which, in turn, affects the ozone residual.

**Ozone decomposition.** Ozone decomposed at 3.2-25.4% per minute depending on the water (Fig. 2.10). Ozone in distilled, deionized, and HPLC water decomposed at <4% per minute, while two tap waters and buffer decomposed ozone at 21, 16.8 and 27.4% per minute, respectively. Ozone decomposes spontaneously in air and water leaving only oxygen, therefore, it is usually generated at the point of application. It is more stable in the gaseous than in aqueous form (Stumm, 1958). The half-life of gaseous ozone in ambient atmosphere is ~12 hrs, while the half-life of aqueous ozone is less than 30 min. The half-life of ozone in distilled or tap water at 20°C is ~20 min (Rosenthal, 1974). However, in double-distilled water, only 10% of the ozone decomposed over a period of 85 min. In this study, the half-life of ozone at 24°C in deionized and tap water was 12 and 6 min, respectively (Fig. 2.10). Since all microorganisms were inactivated in less than 30 seconds of mixing ozone with bacterial cells, therefore, the stability of ozone was not a crucial factor in this investigation.

Masschelein (1982) noted that first-order kinetics generally described the decomposition of ozone. Peeters et al. (1989) used deionized water for their experiments,
which typically supports a first-order ozone decay. In their study on decay of ozone residual in 0.05 M phosphate buffer seeded with *E. coli* (10⁶ cfu/mL), Finch, et al. (1988) found that ozone decomposed at first-order when the concentration was 0.044-0.81 mg/L. In the presence of OH radical scavengers, including certain buffers, the second kinetic order with respect to ozone may be observed (Nadeshkin, 1988). Studies on ozone destruction in mineralized water have suggested that destruction progresses according to a second-order or perhaps a 1.5-order law (Gontelli, 1972).

**Inactivation studies**

The inactivation patterns observed in this study 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 (10⁶ cfu/mL). Bacterial count decreased by 3-6 log in 60 sec. The disinfection rate was fast initially. They concluded that the disinfection kinetics did not follow the pseudo first-order model which is normally assumed to approximate chemical disinfection of bacteria. In a batch-type reaction system, 0.055 mg ozone/L inactivated 3.5 log *E. coli* in 30 sec (Kazadielson et al., 1974). A two-stage action of ozone in the inactivation of *E. coli* was observed. The investigations concluded that ozone acts on microorganisms so quickly that it is practically impossible to measure the time required for 99% kill.

Residual ozone was determined in order to reveal the mechanism involved in the inactivation in the batch reaction (Fig. 2.16 and 2.17). 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 et al. (1977) observed the degree of inactivation was profoundly affected by the initial organism population. These authors detected 4-log reduction occurred when the initial density of *C. parapsilosis* was 1.4x10⁵ cfu/mL, while no observable inactivation took place when the initial density was 1.6x10⁷ 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 biological 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 demand substances are generated during the ozone inactivation process. Finch, et al. (1988) found that 10⁶ *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 (Fig. 2.12 to 2.15). 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. Sporeformers are more resistant to destruction by ozone than non-sporing bacteria with the aerobic sporeformers, such as B. cereus and B. megaterium, being more easily destroyed than the anaerobic spore formers, e.g., Clostridium perfringens and C. baccilliform (Broadwater, 1973; Ito and Seeger, 1980; Seeger, 1978). 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 (Fig. 2.14 to 2.21). However, two different inactivation models exist depending on the applied ozone dose and should be applied at the limited ozone concentration. 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 Hoigne (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 tubing, but ozone was quickly depleted during the holding period (Fig. 2.4). Although this design maximizes bactericidal action of ozone and minimizes ozone depletion by intracellular components, data were only marginally better in describing inactivation patterns than were the data from the batch system (Fig. 2.22 to 2.25).

The reaction system was modified to permit continuous exposure of bacteria to constant concentration of ozone during the treatment. Bacterial cells were held stationary on a membrane filter and ozone was pumped through the loaded filter (Fig. 2.5). Continuous reactor II gave inconsistent data (Fig. 2.26). Components of reactor II, e.g., filter holder (Dekin acetyl resin) and O-ring (Viton) probably have an ozone demand and the distribution of cells on the filter may have been uneven. Refinement of the continuous reactors' design led to the development of reactor III (Fig. 2.6).

Inactivation data were better described by first-order kinetics when using reactor III than any of the previously-tested systems (Fig. 2.27 to 2.30). 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 (Fig. 2.31 to 2.34).

Jorer et al. (1982) reported that the inactivation of E. coli present in wastewater was 1.5 and 3 log unit by 1.4 and 2.2 mg/L ozone, respectively, for a 19-minute 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 (Salmonella 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, decrease >5 log instantaneously. In case of L. monocytogenes, the count decreased >5 log immediately, but only a decrease of 3 log was observed for S. aureus, E. faecalis, and S. 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 not consistent with the findings of this study.

Hunt and Marinas (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 five to seven 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 Marinas (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. They estimated that $4 \times 10^7$ molecules of ozone are required to inactivate 2 log of E. coli cells. 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 convex downward pattern, which show an initial lag period before first-order inactivation, or concave downward kinetics, that is, a rapid initial decline in populations, or 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; Katznelson et al. 1974). Cellular debris from the damaged or lysed organisms shield the surviving E. coli from the effects of ozone (Finch et al., 1988). These authors calculated that $3 \times 10^8$ molecules of ozone was 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 which has a "tail".

Varius explanation for causes of "tailing" during disinfection with ozone were reported. Hoigne (1982) suggested a shielding which 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.
and since the disinfection reaction rate may be decreasing, competing reactions for ozone may be successful in using ozone, thereby shielding remaining viable organisms. It is still unclear whether this shielding effect is common for all microorganisms or if there are differences among different categories of microorganisms.

In this study, log units inactivated (PI) or D-values were used to construct the log-log dose-response plots and compare the ozone efficiency for the inactivation of different microorganisms. Effectiveness of different decontaminants is generally evaluated by comparing the decontaminant concentration x contact time (C*t) data for the inactivation of the organisms to a given level (e.g., 99% inactivation). The “C*t” concept is based on an empirical relationship reported by Watson (1908). Hampson et al. (1998) used C*t (mg/L ozone x minutes) values vs log reduction to compare the effectiveness of ozone in the treatment of different commodities.

In conclusion, ozone inactivates bacteria rapidly in a batch reactor and continuous reactor II and decomposed during the cell inactivation. Survivors plots showed initial rapid inactivation and no further inactivation after 10 sec of exposure to ozone. This phenomenon is believed partially due to the depletion of residual ozone in the reaction mixture. Ozone exerts its action in a few seconds, therefore inactivation kinetics are difficult to perform in a batch and a continuous reactor I. A continuous ozone reactor III was developed to ensure a uniform exposure of bacterial cells to ozone and constant concentration of ozone during the treatment. When using this system, approximated inactivation kinetics that are consistent with general disinfection patterns, were observed. Resistance of tested bacteria to ozone followed this descending order: E. coli O157:H7, P. fluorescens, L. sp.
mesenteroides, and L. monocytogenes. Therefore, more developed designs and additional works are needed to obtain kinetic models.

References


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<th>Water</th>
<th>Distilled&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Deionized&lt;sup&gt;2&lt;/sup&gt;</th>
<th>HPLC&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Buffer&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Tap water I&lt;sup&gt;5&lt;/sup&gt;</th>
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<sup>1</sup> Double distilled water.
<sup>2</sup> Obtained from a Milli-Q system.
<sup>3</sup> HPLC grade water manufactured by Fisher Scientific.
<sup>4</sup> Phosphate buffer (0.05 M).
<sup>5</sup> Tap water I is fountain water in the Department of Food Science and Technology.
<sup>6</sup> Tap water II is tap water in a research lab.

Table 2.1. pH values of different waters.
Figure 2.2. Relationship between U.V. absorbance at 258nm and ozone concentration.

\[ Y = 0.0515645 \times X - 0.0161199 \]

\( R^2 = 0.99349 \)
Oxidation of water by bubbling ozone gas (ca. 5%) from an ozone generator

Ozone measurement by U.V. and Indigo method

Addition of culture into ozonated water

Transfer each sample into neutralizer

Bacterial counting

Figure 2.3. Experimental scheme for the kinetic study by the batch reaction.
Figure 2.5. Continuous reactor II.
Figure 2.6. Continuous reactor III.
Figure 2. Spectrum of counted water scanned by a diode-array spectrophotometer.
Figure 2.8. Ozonation of different water with ozone gas (ca. 5%, v/v) at a flow rate of 29.4 ml/min. Tap I; Fountain water, Tap II; Tap water in the research lab.
Figure 2.9. Ozone stability in phosphate buffer (0.01 M) having different pHs.
Figure 2.10. Ozone decomposition in different waters. No. ozone concentration at initial time, No. residual ozone concentration.
Figure 2.11. Ozone decomposition in deionized water having different initial level of ozone. No; ozone concentration at initial time, N; residual ozone concentration.
Figure 2.12. Survivors plots for the inactivation of *P. fluorescens* (2.5x10⁸ cfu/ml), initially by ozone at pH 5.9 and 25°C. No; count of untreated sample, N; count of treated sample.
Figure 2.13. Survivors plots for the inactivation of E. coli O157:H7 (2.5x10^8 cfu/ml initially) by ozone at pH 5.9 and 25°C. N₀: count of untreated sample, N: count of treated sample.
Figure 2.14. Survivors plots for the inactivation of *L. mesenteroides* (1.3x10^8 cfu/ml, initially) by ozone at pH 5.9 and 25°C. No; count of untreated sample, *N*; count of treated sample.
Figure 2.15. Survivors plots for the inactivation of *L. monocytogenes* (1.8x10^6 cfu/ml, initially) by ozone at pH 5.9 and 25°C. No; count of untreated sample, N; count of treated sample.
Figure 2.16. Changes of residual ozone concentration during inactivation of *L. mesenteroides* (1.2x10^7 cfu/ml) by ozone.
Figure 2.17. Changes of residual ozone concentration during inactivation of *L. mesenteroides* having different inoculum size.
Figure 2.18. Dose-response plot for the inactivation of P. fluorescens (2.5x10^6 cfu/ml, initially) by ozone. No; count of untreated samples, N; count of samples after 30 seconds of mixing with ozone.
Figure 2.19. Dose-response plot for the inactivation of *E. coli* O157:H7 (1.7x10^8 cfu/ml, initially) by ozone. N0: count of untreated samples; N: count of samples after 30 seconds of mixing with ozone.
Figure 2.20. Dose-response plot for the inactivation of *L. mesenteroides* (1.4x10⁸ cfu/ml, initially) by ozone. No, count of untreated sample, N, count of sample after 30 seconds of mixing with ozone.
Figure 2.21. Dose-response plot for the inactivation of *L. monocytogenes* (1.9x10^6 cfu/ml, initially) by ozone. No, count of untreated samples; Ni, count of samples after 30 seconds of mixing with ozone.
Figure 2.23. Survivors plots for the inactivation of *E. coli* O157:H7 (4.3×10⁸ cfu/ml, initially) by ozone in the continuous reactor I. No; count of untreated sample, N; count of treated sample.
Figure 2.24. Survivors plots for the inactivation of L. mesenteroides (2.0×10^8 cfu/ml, initially) by ozone in the continuous reactor. N₀: count of untreated sample, N: count of treated sample.
Figure 2.25. Survivors plots for the inactivation of *L. monocytogenes* (2.4x10^8 cfu/ml), initially by ozone in the continuous reactor I. No; count of untreated sample, N; count of treated sample.
Figure 2.27: Survivors plots for the inactivation of *P. fluorescens* (6.6x10^7 cfu/ml, initially) by ozone in the continuous reactor III. No; count of untreated sample, N; count of treated sample.
Figure 2.28. Survivors plots for the inactivation of *E. coli* O157:H7 (7.6x10⁷ - 2.2x10⁸ cfu/ml, initially) by ozone in the continuous reactor III. No. count of untreated sample, N, count of treated sample.
Figure 2.29. Survival plots for the inactivation of *L. mesenteroides* (1.0x10⁸ cfu/ml), initially by ozone in the continuous reactor III. No: count of untreated sample, N: count of treated sample.
Figure 2.31. Log-log dose-response plot for the inactivation of *P. fluorescens* 
(2.8x10⁷ - 6.9x10⁷ cfu/ml, initially) by ozone in the continuous reactor III.
Figure 2.12. Log-log dose-response plot for the inactivation of *E. coli* O157:H7 (7.6×10⁷ - 2.2×10⁸ cfu/ml, initially) by ozone in the continuous reactor III.
Figure 2.33. Log-log dose-response plot for the inactivation of *L. mesenteroides* by (9.3x10^7 - 1.7x10^8 cfu/ml, initially) ozone in the continuous reactor III.
Figure 2.34. Log-log dose-response plot for the inactivation of *L. monocytogenes* by (8.1x10^7 - 2.0x10^8 cfu/ml, initially) ozone in the continuous reactor III.
CHAPTER 3

INACTIVATION MECHANISMS OF FOODBORNE MICROORGANISMS

Abstract

Injury of *Pseudomonas fluorescens*, *Escherichia coli* O157:H7, *Leuconostoc mesenteroides*, and *Listeria monocytogenes* by ozone was evaluated using a salt sensitivity test. Inactivation and injury by ozone were inversely related. Resistance to ozone and susceptibility to injury were greater in *P. fluorescens* and *E. coli* O157:H7 than in *Leu. mesenteroides*, and *L. monocytogenes*. These differences between gram-positive and gram-negative bacteria suggest that inactivation mechanism may depend on the structure of cell envelop.

Washed bacterial cells on membrane filters were exposed to ozonated water in a continuous reaction system and morphology of ozone-treated cells was examined by a scanning electron microscope. Water containing ~2 mg ozone/L caused profound structural damage to *P. fluorescens* and *E. coli* O157:H7 but cells of *Leu. mesenteroides* and *L. monocytogenes*, remained intact after the ozone treatment.

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A cell suspension of *P. fluorescens* was bubbled with ozone gas, centrifuged, and the absorbance of the supernatant was scanned at 200-850 nm by a spectrophotometer. As the contact time with ozone increased, area of peaks at the wavelengths between 220 and 280 nm increased. Therefore, ozone may cause cell membrane damage and thus release DNA, RNA or other UV-absorbing cellular components.

In conclusion, small concentrations of ozone are more injurious and less lethal to gram-negative than gram-positive bacteria. A mechanism for inactivation of bacteria by ozone was suggested. Ozone reacts with the outer membrane of gram-negative bacteria; this causes cell injury and releases components with appreciable ozone demand. Ozone is consumed in reactions with released components and thus a small amount of the agent remains to inactivate the cell. In case of gram-positive bacteria (e.g., *Listeria*), ozone reacts minimally with cell envelop and sufficient amounts penetrate the cell wall, interact with cytoplasmic material and thus cause appreciable cell death.

Introduction

Although many research groups studied mechanisms of microbial inactivation by ozone, most of these studies produced controversial data. Goldstein and Balchum (1967) and Scott and Lesher (1963) suggested that ozone inactivates microorganisms because it degrades proteins and unsaturated lipids in cell membrane. More recent studies (Hamelin et al., 1978, Takamoto et al., 1992) suggested that ozone targets enzymes and nucleic acids in the cytoplasm.
Many studies were performed on \textit{E. coli} and other microorganisms of importance in water and wastewater treatment (Bringman, 1955; Fisch et al., 1987; Hanelin and Chung, 1975; Ishizake et al., 1987; Komanapalli and Lau, 1996; Prat et al., 1968, Scott and Leshner, 1963). Few mechanistic studies, however, were done on foodborne microorganisms. In most mechanistic studies, researchers exposed microorganisms or cellular fractions to exposed ozone for extended periods and monitored changes to cells by chemical methods (Goldstein and Balchux, 1967; Hanelin et al., 1977, 1978; Ishizake et al., 1987; Komanapalli and Lau, 1996; Mudd et al. 1969; Scott and Leshner, 1963). Our kinetic studies (Chapter 2) showed that most of microbial inactivation occurs within 10 seconds of encounter between ozone and the cells. Therefore, cellular response to ozone should be monitored after brief exposures so that the inactivation mechanism can be elucidated.

A different approach in studying the inactivation mechanism by ozone is followed in this study. We hypothesize that ozone interacts rapidly with cell envelope and causes damage which is manifested in cell injury and death. Consequently, ozone may interact with gram-positive and gram-negative bacteria differently. Additionally, presence of cell capsule may modify appreciably the effectiveness of ozone against the treated bacterium. Two gram-negative bacteria (\textit{P. fluorescens} and \textit{E. coli} O157 H7) and two gram-positives (\textit{Leu. mesenteroides} and \textit{L. monocytogenes}) were investigated.
Materials and Methods

Methods reported earlier chapter (Chapter 2) were followed for production, preparation and measurements of ozonated water and preparation of bacterial cultures.

Inactivation studies

Bacteria were treated with ozone in the continuous reactor type-III (Fig. 2.6), as indicated in the previous study (Chapter 2). Cell suspension (1-2 mL, ~10^6 cfu/mL) was filtered through a ozone-resistant membrane filter (0.45 μm, 25mm, HAWP, Millipore, Marlborough, MA), held in a filter funnel. When the ozone concentration reached a steady state in an ozone absorption flask, a predetermined volume of ozonated water was transferred onto the filter membrane and pumped through by a peristatic pump (Masterflex, Cole-Palmer, Vernon Hills, IL). After the treatment, the membrane filter, loaded with treated cells, was transferred into a peptone dilution tube to stop ozone reaction and to prepare the sample for plate counting. Similarly-treated cells were also examined by the scanning electron microscope.

Cell injury

Sensitivity of bacteria to salt was measured to determine the threshold concentration of salt that inhibit injured cells. Petri plates containing serial concentrations of salt (0.0-6.0 %) were prepared and prewashed cells were diluted and plated on these agar plates. Colonies were counted after 2-3 days of incubation at 26°C for *P. fluorescens* and *L.*
mesenteroides and 37°C for *E. coli* O157:H7 and *L. monocytogenes* to determine the minimum salt concentration which inhibits growth of healthy cells (Fig. 3.1). This minimum salt concentration presumably inhibits growth of injured cells and thus was used in agar to count only healthy cells. Healthy and ozone-injured cells are counted on agar without added salt. Prewashed cells were captured on membrane filter and treated with ozone (0.5-3.0 ppm) as indicated earlier. The loaded filter was transferred into peptone diluent tubes and mixed using a vortex mixer. Cells were diluted by 10 fold dilutions and plated on the agar plates with or without added salt. The difference in colony counts between these two sets of plates is considered the count of injured cells.

**Scanning electron microscopy**

Bacteria on filter membranes were treated with ozone as indicated earlier. For this study, a 0.4 μm membrane filter (HTTP, Isopore filter, Millipore, Marlborough, MA) which is made of bisphenol polycarbonate, was used instead of HAWP, because the membrane offers advantages for electron microscopic analysis. Membrane filters trapping ozone-treated and untreated cells were cryogenically dried at critical point with liquid carbon dioxide. The completely dried membrane filters were coated with gold and palladium. Microscopic analysis was performed by a scanning electron microscope (JEOL JSM-820).

**Spectrophotometric method**

Pre-washed cells in deionized water were bubbled with ozone gas for 30 seconds in order to inactivate cells. Ozone-treated and untreated cell suspensions were centrifuged
at 10,000 rpm for 2 min. Absorbance of UV and visible spectra (200-850nm) by the supernatant was determined using a diode-array spectrophotometer (Hewlett-Packard model 8452A, Chicago, IL).

Microbiological tests

Microbiological methods employed were selected from the Bacteriological Analytical Manual (BAM, 1992) and the Compendium of Methods for the Microbiological Examination of Foods (Vanderzant and Splitsstootsra, 1992).

Results

Cell injury by ozone

Minimum inhibitory salt concentrations for *P. fluorescens*, *E. coli* O157: H7, *Leu. mesenteroides*, and *L. monocytogenes* are 4.0, 7.0, 4.0, and 6.0% (considered 0.5% NaCl initially present in TSAYE), respectively (Fig. 3.1). It is evident that *L. monocytogenes* is the most, while *E. coli* O157:H7 is the least salt tolerant among bacteria included in this study. Agar media containing these salt concentrations were prepared to count healthy (non-injured) bacterial cells.

Cell suspensions were treated with low (0.55-1.0 ppm) and high (1.85-2.53 ppm) ozone concentrations. Differences in survival rates were observed when ozone-treated cells were plated on the nonselective or the salt-containing agar media (Fig. 3.2, 3.4, 3.6, and 3.8). The total viable population, represented by the count on the nonselective media, decreased
rapidly at the first phase of inactivation and did progressively during the extended contact time.

Injury resulting from ozone treatment was appreciable in *Pseudomonas, Escherichia* and *Leuconostoc*, but it was minimal in case of *Listeria*. Injury varied with contact time and ozone concentration. The pattern of changes in injury was similar for *Pseudomonas, Escherichia* and *Leuconostoc* (Fig. 3.3, 3.5, and 3.7). However, *Listeria* did not show a distinct pattern of change in injury (Fig. 3.9). Maximum injury occurred after short contact times at high ozone concentration and after longer contact times when the smaller ozone concentration was used. Therefore, there seems to be an optimum C*t* values for injury of each of the bacteria tested in this study. C*t* values, at which the maximum injury occurred by ozone, was 20, 12, and 29 for *Pseudomonas, Escherichia*, and *Leuconostoc*, respectively. Maximum injury percentage was approximately 76, 60, and 56, accordingly. Gram-negatives tended to suffer more injury by ozone than did the gram-positives.

**Electron microscopical analysis**

Cells on membrane filters were treated with ozonated and non-ozonated water; one set of treated and untreated cells were examined for morphological change by a scanning electron microscope, and the other set was tested for survival and injury. The microbial load on the membrane filter was 5.6, 7.1, 7.0, and 6.9 log for *P. fluorescens, E. coli* O157:H7, *L. mesenteroides*, and *L. monocytogenes*, respectively. With ~2 mg/L ozone, populations of all tested bacteria, except *L. monocytogenes*, decreased ~2 log within 6 seconds; count of *L. monocytogenes* decreased by 2.8 log. Injury by ozone was 0.29 and
0.36 log in *P. fluorescens* and *L. mesenteroides*, while it was 0.12 and 0.02 log in the case of *E. coli* and *L. monocytogenes*, respectively. Injury percentage for gram-positives agrees with that obtained in previous experiments at similar C* values (Fig. 3.6 and 3.8), although the treated inoculum size was ca 1 to 1.3 log units lower than the previous experiment (Table 3.1). However, in the case of gram-negatives, *P. fluorescens* and *E. coli*, when 2 and 0.8 log lower initial inoculum were used for the reaction, 20 and 40% lower injury was obtained, respectively.

When *P. fluorescens* was treated with 1.85 mg/L ozone for 5.7 sec, the viable count decreased by 1.7 log, therefore, 98% of population was killed by this ozone treatment. Thus, most cells on the filter membrane are dead. Plate counting was performed immediately after the ozone treatment for the cells on the membrane filters. Cells of *P. fluorescens* appeared in clumps or aggregates of rods and each cell maintained its distinct morphology (Fig. 3.10a). Ozone destroyed the cellular structure and only cell debris remained on the membrane (Fig. 3.10 b). When cells of *E. coli* O157:H7 were treated with ca. 2.2 mg/L aqueous ozone, ~99% of population were inactivated. Micrographs of treated and untreated cells revealed a morphology similar to that of *Pseudomonas* (Fig. 3.11 a and b).

In the case of *L. mesenteroides*, 2.3 mg ozone/L inactivated 98.7% of cell population. The untreated bacterium (suspended in water) appeared in clumps of spherical cells covered with mucoid material (Fig. 3.12 a). Although ozone-treated cells were mostly dead but cells remained intact and appeared with distinct morphology (Fig. 3.12b). Treated cells also appeared less bulky and lost the mucoid material.
When *L. monocytogenes* was treated with 2 mg ozone/L for 5.7 sec, the count decreased by 2.8 log, i.e., 99.8% destruction. Cells on the filter membrane appeared in groups of short rods (Fig. 3.13a). The inactivation mechanism of *Listeria* appeared similar to that of *Leu. mesenteroides*; ozone did not cause any apparent deformation of the cell structure (Fig. 3.13b).

Leakage of cell content

*P. fluorescens* cell suspension was bubbled with ozone gas for 30 seconds and release of UV-absorbing cellular component (e.g., proteins and nucleic acids) was measured in the supernatant. Supernatant from untreated cells showed no peaks, although some blunt and wide area was present below 280 nm (Fig. 3.14). When cells were treated with ozone, supernatant gave distinct peak at 257 nm (Fig. 3.15). The peak area below 280 nm also increased in size.

Discussions

Cell injury

Ray (1979) noted that a bacterial population, which survives sublethal physical or chemical treatments during food processing, is composed of injured and healthy or normal cells. Injured cells may suffer damage to surface structures such as lipopolysaccharide layer in gram-negative (Hambleton, 1971; Hurst, 1977), teichoic acid in gram-positive bacteria (Hoover and Gray, 1977; Jonson et al., 1978) and the cytoplasmic membrane (Allwood 126
and Russell, 1970; Calcott and MacLeod, 1975; Strange and Postgate, 1964). Damage to injured cells can be repaired in a nutritionally adequate environment, but not in the presence of selective agents (Ray, 1979).

Injured cells grow poorly on selective media such as those used normally in sanitary microbiology, thereby underestimating the density of surviving organisms (Camper and McFeters, 1979; McFeters et al. 1978; Stuart et al. 1977). Therefore, if ozone causes appreciable cell injury, indicator and pathogenic microorganisms from areas sanitized with this agent may escape detection. In this study, injury of selected spoilage and pathogenic foodborne bacteria by ozone was evaluated. The percentage of injured cells among the survivors varied depending on the tested microorganisms and the applied ozone concentration (Fig. 3.2, 3.4, 3.6, and 3.8). All microorganisms except *L. monocytogenes* showed maximum injury rate at the intermediate C*ₚ* (ppm x sec) value between 12.0 and 29. The injury maximum was at 3 C*ₚ* value for *L. monocytogenes*. Ozone (1.9-2.6 ppm) caused the greatest injury to *P. fluorescens* and the least to *L. monocytogenes* (Fig. 3.3, 3.5, 3.7, and 3.9).

Duitschaever and Jordan (1974) studied thermal injury of *Enterococcus faecium* and its resistance to heat and sodium chloride. They suggested that cellular defects of injured cells are located in the cell envelope since injury is commonly manifested in increased leakage of cell content, increased salt sensitivity and an altered response of recovered cells to salt. Therefore, increased salt sensitivity of gram-negative microorganisms treated with ozone in this study may resulted from damage to outer membrane-containing oxidizable lipid.

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Scott and Lesher (1963) investigated the recovery of bacterial growth after ozone treatment. They reported that treated cells did not resume growth on salts and glucose-containing media but were able to grow on rich media. Legaron (1984) noted that when a large part of the membrane barrier is destroyed, the bacterial or protozoan cells lyse, which results in their destruction. However, if the membrane barrier is only slightly damaged, it could be repaired by the bacterial cell, which may explain the revival phenomena that sometimes are noted.

Electron microscopical analysis

Electron microscopical examination of ozone-treated cells elucidated that gram-negative microorganisms (i.e., *P. fluorescens* and *E. coli* O157:H7) lost their skeletal structure, probably due to the degradation of the outer membrane. Gram-positive bacteria (i.e., *Leu. mesenteroides* and *L. monocytogenes*) were killed by ozone, however, they maintained most of their cellular morphology. Therefore, inactivation of bacteria by ozone may follow different mechanisms depending on the structure of cell envelop.

*Leu. mesenteroides* produces a characteristic slime (dextran) formed from sucrose (Bergey’s manual, 1974). The electron microscopic analysis showed that ozone removed the slime layer which cells maintained during preparation of the suspension (Fig. 3.10 to 3.13). The cell wall contains a peptidoglycan layer which is thick in gram positive bacteria and thin or absent in gram-negatives. Peptidoglycan provides rigidity to the cell wall of gram-positive bacteria but the scarcity of this substance makes gram-negative bacteria susceptible to mechanical breakage. This study shows that ozone degraded the outer
membrane of P. fluorescens and E. coli O157:H7, and caused the disintegration of cell structure which resulted in cell lysis (Fig. 3.10 and 3.11). However, as shown in microscopic pictures, gram-positive bacteria such as L. monocytogenes and L. mesenteroides kept their shape in spite of ozone reaction (Fig. 3.12 and 3.13).

The precise chemical reactions that cause microbial death after exposure to ozone is still not clear. However, inactivation mechanisms suggested by various researchers may follow all modes of microbial inactivation as categorized by Davidson and Branen (1981), i.e., (a) reaction with the cell membrane, causing increased permeability and loss of cellular constituents; (b) inactivation of essential enzymes; and (c) destruction or functional inactivation of genetic material.

According to Staelhein and Hoigne (1985), ozone reacts with various chemical compounds in aqueous systems by two different and coexisting modes, direct reactions of molecular ozone and a free radical mediated destruction mode. These reactions may cause a general oxidation of susceptible cellular components on the surface of bacterial cell (Legeron, 1984). According to Caraccioolo (1998), ozone destroys bacteria by rupturing the cellular membrane and dispersing the bacterial cytoplasm in the water. Many workers (Heath, 1978; Mead, 1976; Peters, 1982) showed that lipids extracted from subcellular materials or microsomal fractions were sensitive to ozone-induced oxidation.

Suggested mechanism

Findings from this and previous studies may be sufficient to suggest a mechanism for action of ozone against bacteria. Ozone, at small concentration (<1ppm), reacts with
the outer membrane of gram-negative bacteria. This reaction causes limited damage to the outer membrane which is manifested in cell injury. Damage to the outer membrane releases components with appreciable ozone demand. Some ozone is consumed in reactions with released components and thus a small amount of the agent interacts with the inner membrane and inactivates the cell. Ozone at a higher concentration (~2ppm) causes substantial damage to both outer and inner membranes which results in cell lysis. Components released from lysed cells may neutralize residual ozone and thus cells within clumps (or those protected by other means) may survive the treatment.

In case of gram-positive bacteria (e.g., Listeria), ozone reacts minimally with cell envelop and sufficient amounts of the agent penetrate the cell wall, interact with cytoplasmic material and thus cause appreciable cell death. Presence of a slimy layer around the cell, as the case in Leuconostoc, protects inner membrane against oxidation by ozone. Therefore, inactivation kinetics of these slime-producing bacteria appear similar to that of gram-negatives.

References


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<table>
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<tr>
<th></th>
<th>C*t values</th>
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<td>63.7</td>
<td>7.88</td>
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<tr>
<td></td>
<td>13.52</td>
<td>24.1</td>
<td>7.08</td>
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<td>14.54</td>
<td>56.3</td>
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<tr>
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<td>8.5</td>
<td>8.34</td>
<td>Fig. 3.8</td>
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<tr>
<td></td>
<td>11.25</td>
<td>4.5</td>
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*C*t values: ozone concentration-contact time (ppm x sec)
Injury (%) = 100 x (count of healthy plus injured cells - count of healthy cells)/count of healthy plus injured cell

Table 3.1. Injury (%) of foodborne microorganisms containing different initial inocula during the ozone treatment.
Figure 3.1. Determination of minimum salt concentration to inhibit growth of healthy cells.
Figure 3.2. Ozone inactivation and injury of P. fluorescens (2.8 x 10^7 cfu/ml, initially) in the continuous reactor III. No; count of untreated sample, N; count of treated sample. H; healthy cells, I; injured cells.
Figure 3.3. Decrease in count and percent injury of *P. fluorescens* (2.8x10^7 cfu/mL, initially) during ozone treatment at different concentration-time (C*t) values. No; count of untreated sample, N; count of treated sample.
Figure 3.4. Ozone inactivation and injury of E. coli O157:H7 (7.6x10^7 cfu/ml, initially) in the continuous reactor III. No: count of untreated sample, N: count of treated sample. H: healthy cells, I: injured cells.
Figure 3.5. Decrease in count and percent injury of E. coli O157:H7 (7.6×10⁷ cfu/ml, initially) during ozone treatment at different concentration-time (C^t) values. N₀, count of untreated sample; N, count of treated sample.
Figure 3.6. Ozone inactivation and injury of *L. mesenteroides* (1.0×10⁶ cfu/ml, initially) in the continuous reactor III. No; count of untreated sample, N₁; count of treated sample. H₁; healthy cells, I₁; injured cells.
Figure 3.7. Decrease in count and percent injury of *L. mesenteroides* (1.0×10⁶ cfu/ml, initially) during ozone treatment at different concentration-time (*C*⁻¹) values. No; count of untreated sample, N; count of treated sample.
Figure 3.8. Ozone inactivation and injury of *L. monocytogenes* (2.3x10^9 CFU/ml, initially) in the continuous reactor III. No; count of untreated sample, N; count of treated sample. H; healthy cells, I; injured cells.
Figure 3.9. Decrease in count and percent injury of *L. monocytogenes* (2.3 x 10⁸ cfu/ml, initially) during ozone treatment at different concentration-time (*C*t) values. No; count of untreated sample, N; count of treated sample.
Figure 3.10. Micrographs from the scanning electron microscope illustrating, water-treated (a) and ozone-treated (b) *Pseudomonas fluorescens* cells on membrane filters.
Figure 3.11. Micrographs from the scanning electron microscope illustrating, water-treated (a) and ozone-treated (b) *Escherichia coli* O157:H7 cells on membrane filters.
Figure 3.12. Micrographs from the scanning electron microscope illustrating water-treated (a) and ozone-treated (b) *Leuconostoc mesenteroides* cells on membrane filters.
Figure 3. (3) Micrographs from the scanning electron microscope illustrating water-treated (a) and ozone-treated (b) *Listeria monocytogenes* cells on membrane filters.
Figure 3.14: Spectrum of supernatant of untreated *Pseudomonas fluorescens* cell suspension.
Figure 3.15. Spectrum of supernatant of *Pseudomonas fluorescens* after ozone treatment for 30 seconds.
CHAPTER 4

USE OF OZONATION TO DECONTAMINATE LETTUCE

Abstract

Ozone effectiveness on lettuce was compared with that of chlorine. Ozone inactivated mesophilic and psychrotrophic bacteria by 0.84 and 1.2 log_{10} cfu/g at 1.3 mM ozone gas in 1.5 min and by 1.38 and 1.8 log_{10} cfu/g in 3 min, respectively. At 1 mM of chlorine, the average inactivation of mesophilic and psychrotrophic bacteria was 1.38 and 1.8 log_{10} cfu/g, respectively, with 3 min of contact time. Inactivation was 1.95 and 2.85 at 2 mM of chlorine with same contact time, respectively. It may be concluded that ozone is comparable to chlorine as an antimicrobial agent for use in washing of minimally-processed vegetables.

In order to improve the antimicrobial effectiveness of ozone (O₃) in fresh lettuce processing, different ozonation procedures were tested. Lettuce was shredded into pieces of approximately 2.5 x 2.5 cm. Shredded lettuce was treated with gaseous ozone or water into which ozone was bubbled. For effective delivery of ozone, stirring (low and high speed) or sonication was applied during ozonation. Additionally, stomaching was applied.
before and/or after ozone treatment.

Washing with water only decreased total count on shredded lettuce by 0.74, 8.83, 1.02 and 1.03 log cfu/g for low-speed stir, stir plus stomaching, stir plus sonication and high-speed stir, respectively. Differences in count among these delivery methods were not significant (P>0.05).

Direct delivery of gaseous ozone into a treatment chamber containing the shredded lettuce decreased the total count by 0.85 log cfu/g. When vacuum was applied followed by flushing with ozone gas, the total count on lettuce decreased by 0.96 log cfu/g.

When gaseous ozone was bubbled into the lettuce-water mixture, it enhanced microbial inactivation. When ozone gas bubbling was applied during sonication and high-speed stir and before stomaching, inactivation rates were 1.41, 1.88 and 1.93 log cfu/g, respectively. When lettuce was stomached before and after ozone bubbling, count of natural flora on lettuce decreased by 2.89 log cfu/g.

It may be concluded that bubbling gaseous ozone in water was better than other ozonation methods. Efficient ozone delivery to microorganisms on lettuce requires combination of ozone bubbling and high-speed stir.

Introduction

In 1997, A US expert panel self-affirmed ozone as a generally recognized as safe (GRAS) substance (Graham, 1997); this encouraged broader use of this gas in the food industry. As consumption and demand for minimally-processed fresh (MPP) vegetables
increased, the risk of microbial spoilage and contamination by pathogenic microorganisms in these products also increased.

Numerous potentially pathogenic bacteria have been detected in MPF vegetables (Table 4.1). Pathogenic organisms of most concern in these products are pathogenic *Escherichia coli*, *L. monocytogenes*, *Shigella*, *Salmonella*, and hepatitis A virus. These organisms cause disease which were linked to the consumption of contaminated fresh vegetables (Table 4.2).

Chlorine compounds are effective disinfectants against many foodborne microorganisms (Table 4.3). Therefore, chlorine is widely used for the disinfection of MPF vegetables, such as cut salad. Chlorination, however, causes the formation of carcinogenic trihalomethane (THM) in food (Brunn, 1973; Page et al., 1976; Wei et al., 1985). Additionally, low levels of chlorine usually are ineffective against certain bacteria, protozoan cysts, worm eggs, and viruses (Beuchat and Brackett, 1990; Keswick et al., 1984). Chlorine, even when used at low concentration, may cause taste and odor defects in treated products. Ozone is occasionally superior to chlorine in inactivating microorganisms in the pure water, buffer, and wastewater (Kessel et al., 1943; Korich et al., 1990; Scarpino et al., 1972; Selke et al., 1977). However, limited studies were done to test ozone as a disinfectant in fresh vegetable processing.

For effective use in food processing, optimum ozone concentration, contact time, and other treatment conditions should be well defined. Because it is highly reactive, organic matter in the food may compete for ozone with the microorganisms being disinfected. Therefore, inactivation kinetics of ozone vary with the treated commodity.
Objectives of this investigation are (a) to determine the disinfecting power of ozone against natural contaminants in a food, and (b) to maximize the effectiveness of ozone against these contaminants by optimizing its use. Fresh lettuce was tease in this study.

Materials and Methods

Ozone vs chlorine in lettuce processing.

Lettuce. Iceberg lettuce was purchased from the local supermarket. Lettuce was trimmed of discolored and wilted portions. Intact lettuce heads were cut into ~2.5x2.5-cm pieces and mixed well. Samples (25g each) were weighed in stomacher bags in preparation for ozone treatment and microbiological analysis. Lettuce cutting and sample preparation were done aseptically.

Ozone and chlorination demand-free glassware and water. All glassware were washed with a mild detergent and thoroughly rinsed with hot tap water and deionized water (Milli-Q system, Millipore Corp. model OM-140). They were then autoclaved and dried to remove volatile organic compounds. Ozone demand-free water was prepared by ozonating deionized water (Fig. 2.1). The water was then autoclaved at 121°C for 15 min to remove residual ozone and stored in sealed ozone demand-free glass container until needed (Korich et al., 1990).

Ozone production. Ozone (2.0 mM; ~5%, w/v; ~7%, w/w) was produced from purified, extra dry oxygen by an ozone generator (U.S. Filter/Polymetrics T-816, San Jose, CA). The amount of ozone produced by the generator and that available for the treatment

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were determined as indicated later.

**Aqueous ozone.** Ozone water was obtained by bubbling ozone ~4% (wt/wt, in oxygen carrier gas) at a predetermined flow rate (Fig. 2.1). A stainless-steel sparger with 10 µm pore size (Solvent Inlet Filter, Fisher Scientific, Fair Lawn, NJ) was used for bubbling ozone in water. Equilibrium was attained when absorbance at 258 nm remained relatively constant.

**Aqueous chlorine.** Chlorine solutions were prepared by adding predetermined volumes of a 5% sodium hypochlorite solution to a chlorine demand-free sterile water.

**Measurement of ozone.** Ozone concentration in ozonated water was determined by UV spectrophotometry (Badger and Hoigne, 1981). UV absorption at 258 nm ($A_{258}$) was measured in a spectrophotometer (Spectronic 1201, Milton Roy Co., Rochester, NY), and values were converted to ozone concentrations using an equation relating indigo method (Badger and Hoigne, 1981) measurements to $A_{258}$ values. Whenever ozone could not be measured directly in the spectrophotometer, the indigo method was used. Ozone decolorizes indigo trisulfonate (Aldrich Chemical Co., Inc., Milwaukee, WI) and resulting changes in color are measured at $\text{pH} < 4$ in the spectrophotometer, set at 600 nm.

**Ozone and chlorine contact system.** The sample of shredded lettuce in the stomacher bag was diuted 1:20 with ozone or chlorine demand-free water (Fig. 4.1). In experiments, ozone gas was delivered into the stomacher bag through a porous sparger (pore size: 10 µm). After the treatment, a neutralizer solution was added and bag contents were stomached. In some experiments, a beaker was used instead of a stomacher bag and

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the water-lettuce mixture was stirred using a stirrer (Radiometer, Copenhagen, Denmark). For chlorine treatments, a chlorine solution was mixed with the shredded lettuce into the stomacher bag and the mixture was held for a predetermined time.

**Packaging.** Untreated, and chlorine- or ozone-treated lettuce (130-140 g), were packaged under a modified atmosphere, to mimic the commercial practice. Samples were stored at 5°C for 5-12 days.

**Neutralization.** Sodium thiosulfate (0.1 N) stock solution was prepared by dissolving 25 g of Na₂S₂O₅·5H₂O (Sigma Chemical Co., St. Louis, MO) in 1 L of freshly autoclaved distilled water (Rand et al., 1975). The solution was used to neutralize residual ozone and amounts used varied depending on the estimated ozone concentration. When feasible, neutralizer was added to the reaction mixture in amount sufficient to decrease A₂₅₄ to zero. According to preliminary data, amounts of neutralizer added to the solutions had no interfering effect on absorbance at 258nm, nor an adverse effect on treated microorganisms.

**Microbiological analyses.** When the lettuce was ozone-treated in a stomacher bag, the mixture was diluted serially and dilutions were plated, in duplicates, on plate count agar (Difco). Plates were incubated at 37°C for mesophilic, and at 5°C for psychrotrophic counts.

**Challenge study**

**Shredded lettuce.** Iceberg lettuce heads were reduced to proper sample size by radially cutting into wedges. They were cut further into pieces, ~2.5 x 2.5 cm squares,
using an electrical knife. Shredded lettuce was washed with 20 times of its weight sterile deionized water and then spun to remove excess water. Preparation of lettuce was done aseptically.

Inoculum. *P. fluorescens*, a common lettuce spoilage bacterium, was inoculated into the lettuce and inactivation kinetics were studied. *P. fluorescens* was obtained from the culture collection at The Ohio State University and the inoculum was prepared as reported in an earlier section. The stock culture was transferred in nutrient broth which was incubated at 26°C for 24 hr; this was followed by a minimum of three successive transfers. After the inoculation, cells were harvested by centrifugation at 3,000 x g in a refrigerated (4°C) centrifuge (Sorvall RC-5B Superspeed Centrifuge, DuPont Instruments, Wilmington, DE) and washed twice in 0.1 M phosphate buffer solution (pH 7) to a final concentration of 1.3 x 10^6 CFU/ml. Suspensions were stirred using a glass rod and a mechanical stirrer (Vortex, Fisher Scientific Industries, Inc., Bohemia, NY) to minimize cell clumping. The total bacterial count was estimated by measuring A_{660} and calculating approximate count from the standard curve for absorbance vs bacterial count.

The 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 done to determine the cell density of the suspension which is sufficient to give an initial population of ~10^6 CFU/g of lettuce. Shredded lettuce was dipped in the inoculation solution, spun using vegetable spinner and packaged aerobically in a stomacher bag (65 μm (2.6 mm) thickness, Seward, London, UK). Some of the shredded lettuce was packaged under a modified atmosphere (20% CO₂, 80% N₂) using a vacuum packaging machine.
(Sipromac model MC-30, St. Germain, Canada), the inoculum was injected through a septum, and package contents were shook to distribute microorganism on the lettuce surface evenly. Packaging material, in this case, was S50d Cryovac (polyvinylidene chloride) bags (W.R. Grace & Co., Simpsonville, SC). Inoculated lettuce samples were kept in the refrigerator overnight or 10 days to allow attachment of cells on the lettuce surface.

Ozonation of lettuce. The experimental apparatus was similar to that used in the previous study (Fig. 4.1). Inoculated lettuce (ca. 25g) was immersed into ozonated water having different ozone concentration and mixed for 1 min. Ozonated water and lettuce mixture was transferred into a stomacher bag and prepared for microbiological testing. Total microbial count was determined using a method mentioned earlier.

Ozonation and delivery method

Lettuce. Iceberg lettuce was shredded into small sizes (~2.5 x 2.5 cm) using an electrical knife and mixed thoroughly to make samples homogeneous and samples (25-g) were treated with ozone as indicated later.

Ozone contact system. The experimental procedure followed the scheme in Fig. 4.2. Lettuce was treated with ozonated water, ozone bubbled in water, or gaseous ozone. Treatment conditions were as follows: exposure time, 5 min; flow rate of gaseous ozone, 0.5 L/min; ozone concentration in feed gas, 2 mM (4.93%, v/v). Untreated and water-washed (under conditions similar to ozonation treatment) lettuce samples were used as controls. Shredded lettuce was mixed with water, ozonated water, or water bubbled with
ozone, at 1:20 w/v dilution, and the following delivery conditions were tested (Fig. 4.3) (a) low speed (200 rpm) stirring, (b) sonication during low speed stirring, (c) low-speed stirring followed by stomaching for 2 min, and (d) high-speed stirring (300 rpm).

Lettuce was treated with gaseous ozone as follows. Unwashed or water-washed lettuce was treated with gaseous ozone in the reaction chamber (Fig. 4.1). Additionally, reaction chamber containing unwashed lettuce was vacummed and then filled with gaseous ozone.

Microbiological test. A treated or untreated sample (25g) was mixed with water (1:10, w/w) in a stomacher bag and homogenized for 2 min. The mixture was diluted using series of 10-fold dilution and plated in duplicate before counting. Plates were incubated at 37°C for total plate count.

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

Lettuce treatment by ozone and chlorine

Dipping lettuce in water. Water containing different concentrations of ozone was used for the lettuce disinfection. Lettuce was dipped in the ozonated water and the mixture was stirred gently with a stirring rod. Ozone, at 0.1 mM, decreased the natural flora by ~70 %, and water (without ozone) decreased the count by only 35% (Table 4.4). When ozone concentration increased from 0.1 to 2.0 and 0.37 mM, no additional inactivation was observed.

Chlorine, at 0.1 and 0.17 mM, decreased the natural flora on lettuce by 49 and 22%, respectively. Therefore small amount of natural contaminants on lettuce were eliminated by water and chlorine. The average initial count of the lettuce was 4.5 Log cfu/g. About 70% of population decreased with 0.1 mM (~5ppm) ozone and ca. 50% decreased with 0.1 mM (~7ppm) chlorine. Therefore, ozone may be comparable to chlorine for the inactivation of natural contaminants in lettuce.

Lettuce was treated with ozonated water at 7 and 24°C (Table 4.5). Ozone was more effective at the higher than the lower treatment temperature. When lettuce was treated with chlorine, inactivation rate was higher at lower water temperature (Table 4.5)

Lettuce was treated with water, chlorine water (0.2mM), and ozonated water (0.2 mM), packaged under a modified atmosphere, stored in the refrigerator, and psychrotrophic bacteria were counted (Fig. 4.4). Water, chlorine and ozone caused modest and comparable decreases in population of natural flora on lettuce. Water treatment removed ~0.5 log,
whereas chlorine and ozone treatment decreased the population by -0.7 log. The psychrotrophic flora increased during 11 days of refrigerated storage of lettuce from all treatments. At the end of the storage period, counts in the control and the water treatment was $10^8$ cfu/g and that in chlorine and ozone treated lettuce was 7.5 log cfu/g.

**Chlorine and ozone treatment in a stomacher bag.** Chlorine solution at 1 mM inactivated mesophilic and psychrotrophic natural contaminants on fresh lettuce by 1.4 and 1.8 log cfu/g, respectively (Fig. 4.5). At 2 mM chlorine, counts of mesophilic and psychrotrophic microorganisms decreased 1.96 and 2.85 log cfu/g, respectively. Inactivation of natural flora on lettuce by ozone increased as the exposure time increased (Fig. 4.6). Ozone concentration used for lettuce treatment was 1.3 mM ozone in the oxygen-ozone mixture. With 90 seconds of contact time, mesophilic and psychrotrophic microorganisms were inactivated by 0.84 and 1.38 log cfu/g, respectively (Fig. 4.6). When the contact time increased up to 180 seconds, the decrease in count reached up to 1.2 and 1.8 log. The experiment was repeated at longer contact time, and results were compared with those of the control treatment (Fig. 4.7 and 4.8). 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 to that of mesophilic microorganisms, more than 4 log were inactivated in 5 min of ozone treatment. However, there was not any considerable decrease in bacterial count by oxygen. Therefore, it is evident that the inactivation occurs due to ozone alone.
Challenge study

Lettuce was inoculated with *Pseudomonas*, dipped in ozonated water and a gentle turbulence was applied by a stirring rod. Count of *P. fluorescens* decreased as the ozone concentration increased, however, only 80% of population was killed with ~10 mg/L ozone. Inactivation rate was faster for lettuce which was dipped in cell suspension and packaged aerobically than for lettuce which was packaged before inoculation with the *Pseudomonas* culture.

Decontamination of lettuce with high bacterial count by ozone was tested. Shredded lettuce was dipped in a cell suspension of *P. fluorescens* and packaged aerobically (Ino + AP) or anaerobically (vacuum), or packaged before it was inoculated through a septum (MAP + Ino). Lettuce was stored for one or 10 days at the refrigeration temperature and treated with ozonated water with a manual mild agitation (Table 4.6). After one day storage at refrigeration temperature, counts were 5.42 log cfu/g for MPA+Ino and 6.69 log for Ino+AP. Microbial load on lettuce stored for 10 days under different packaging conditions was 6.8-7.8 log cfu/g.

The inactivation rate was not different in the ozone treatment for lettuce stored under different packaging methods. The ozone inactivation rate of one day stored samples was ~10% higher than that of 10-day stored samples. Inactivation percentage for Ino+AP and MAP+Inp was 83 and 80% at one day storage, and 72 and 67% in case of 10 day samples, respectively. Approximately 63.75% of population was inactivated with ~6 ppm of ozone and ~80% of population was inactivated with ~10 ppm ozone. Use of ozonated water with mild agitation was much less effective for the lettuce disinfection than bubbling
ozone into water-lettuce mixture in a stomacher bag plus stomaching. Therefore, it is evident that ozonation and ozone delivery methods are crucial factors to consider in order to achieve efficient disinfection.

**Effective ozonation delivery**

Effective ozonation and distribution methods should be explored to maximize the antimicrobial action of ozone and optimize ozone use. Therefore, objectives of following experiments are to (a) define conditions for effective ozonation process of fresh lettuce, and (b) compare different ozone delivery methods for maximum decontamination of lettuce.

Lettuce used in this experiment contained 4.5-6.3 log cfu/g of total plate count. Water alone, ozonated water and water into which ozone was bubbled removed 0.9, 1.1, and 1.6 log cfu/g, respectively (Fig. 4.10). 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).

Inactivation of natural contaminants on lettuce by ozone varied with the delivery method. The population of natural microflora on lettuce decreased 0.9 log for low-speed stir, 1.1 for stir plus sonication, 1.4 for stir and stomaching and 1.3 for high-speed stir (Fig. 4.11). A significant difference (P<0.05) in microbial inactivation between low-speed stir and low-speed stir plus stomaching was detected.

The interaction effect between ozonation and delivery methods was not significant (P>0.05). The delivery method interacted better with bubbling ozonation than ozone water.
treatment to increase the inactivation of natural contaminants on lettuce. Therefore, in order to find out the best combination of ozonation and delivery methods, one way of analysis of variance for delivery methods and ozonation methods was performed (Table 4.7). 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 inactivation rate was 1.93 and 1.88 log in count, respectively. 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 (Fig. 4.12). Initial microbial load for lettuce used for high-speed stir treatment was 3.8 log and for low-speed stir was 4.9 log. 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 minutes of ozonation. Rate of inactivation during high speed stir treatment diminished after 3 min. while in low-speed stir treatment, it increased progressively in 5 min. When the contact time increased, concentration of residual ozone also increased for both stir treatments. However, rate of ozonation in lettuce-water mixture was smaller when low instead of high speed stir was used. Residual ozone was 33 and 7 ppm after 5 minutes of ozonation which was accompanied with high- and low-speed stir, respectively.
The feasibility of measuring ozone spectrophotometrically in a lettuce-ozonated water mixture was tested. Shredded lettuce and deionized water (1:20 v/v) were mixed in a stomacher bag and shaken at 150 rpm for 30 min by an orbital shaker (Fisher Scientific). The water phase of the mixture was filtered through a microfilter (0.45 μm). Absorption spectrum of filtered aliquot was scanned in a diode-array spectrophotometer (Hewlett-Packard model 8452A, Chicago, IL). The spectrum had a peak at 258 nm which is similar to that obtained with ozone in water. Therefore, residual ozone was determined during lettuce treatment by a chemical (indigo) method.

The decrease in natural contaminants on lettuce were 0.5 log and 0.85 log for water-washed and unwashed lettuce, which were treated with gaseous ozone, respectively (Fig. 4.13). 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

Sanitizing agents must decrease populations of a given test organism at least 5 logs during 30 seconds of exposure at ambient temperatures before the particular agent is deemed effective. According to our previous study (Chapter 3), ozone is clearly an effective sanitizer with great potential applications in the food industry. Ozone, however, is far less effective in decontaminating food surfaces than food-contact or equipment
surfaces. This study addresses the feasibility of using ozone in lettuce processing. Attempts were made to enhance the antimicrobial action of ozone when applied to lettuce processing.

Feasibility of decontamination of lettuce by ozone.

Decontamination of produce by ozone depends on number and kind of contaminating microorganisms, physiology of vegetables, reactor design (e.g., bubbles surface area and turbulence), water quality, temperature, pH and other factors. When ozonated water, without turbulence, was used for the lettuce treatment, minimal elimination of contaminants was observed (Table 4.4, 4.5 and 4.6). Under these conditions, ozone and chlorine were equally poor disinfecting agents. This observation is consistent with the findings of Brackett (1987), who reported that chlorine concentration of less than 50 mg/L in wash water did not kill *L. monocytogenes* in phosphate buffer. This author obtained 1 and 2 log decreases of *L. monocytogenes* when Brussels sprout were dipped in water and in 200 ppm chlorine solution for 30 sec, respectively. Hurst (1993) also noticed that decontamination by dipping food in ozonated water was inadequate. In his study, dipping poultry carcass, which was inoculated with *Salmonella typhimurium*, in ozonated saturated water did not inactivate the pathogen.

Organic matter reacts readily with ozone and decreases its sanitizing power (Block, 1982). Therefore, ozone becomes less effective when used to inactivate bacteria in MPF vegetables rather than in a pure cell suspension. To achieve considerable microbial inactivation, a longer contact time is needed in the former than the latter case. Haines
(1937) observed that large amount of ozone was required to preserve food in which contamination has already begun to develop. Kass (1936) reported that relatively low concentrations of ozone were effective to disinfect meat carrying small numbers of bacteria.

Temperature during ozonation. Ozone inactivated contaminants on lettuce more efficiently when it was applied at higher than refrigeration temperatures (Table 4.5). Ewell (1941) found that antimicrobial action of ozone was similar at 4 and 20°C. However, in an earlier study (Kass, 1936), ozone was more effective against meat microflora at low than at high temperatures. Ozone is relatively stable in water at refrigeration temperatures but it decomposes at a faster rate when the temperature increases (Sease, 1976). The antimicrobial action of ozone, however, is associated with some of its decomposition products, i.e., HO radical (Struebig and Heigl, 1985). Decomposition of ozone also increases with the presence of organic matter and minerals in the reaction mixture. Therefore, although ozone is relatively stable and effective against cell suspensions at low temperatures, optimum temperature for ozonation of complex food system may vary.

Ozone bubbling. The bubbling ozone in water-lettuce mixture inactivated ~2 log of natural flora (Fig. 4.6). Chlorine inactivated more microorganism than ozone did (Fig. 4.5). However, concentration and treatment duration and conditions for ozone and chlorine were different; ozone gas at 1.3 mM in oxygen-ozone mixture was bubbled for 90 and 180 seconds, whereas chlorine at 1 and 2 mM was used for 3 min. When lettuce was exposed to ozone bubbling in a stomacher bag with 1.3 mM ozone at 24°C for 5 min, populations of mesophilic and psychrotrophic microorganisms decreased 3.9 and 4.6 log, respectively (Fig. 4.7 and 4.8). This long exposure time is likely to be impractical in food.
applications.

Challenge study. Lettuce was inoculated with *Pseudomonas fluorescens* (ca. 10^8 cfu/mL), refrigerated for one or 10 days to allow different degree of flora buildup, and immersed in ozonated water (ca. 3-10 ppm). Ozone treatment decreased the count of *Pseudomonas* <1 log in lettuce stored for 1 and 10 days (Fig. 4.9 and Table 4.6). Therefore, dipping lettuce in ozonated water should be replaced with bubbling ozone treatment for the efficient ozone use.

Montecalvo et al. (1998) used ozone to inactivate *Shigella* Coliform, and *E. coli* O157:H7, which were used to inoculate lettuce. Count of *Shigella* decreased 3-4 log (count was 9.2 log, initially) in 3 min by 0.15 and 5 log by 0.3 mg ozone/L. These authors observed 3-4 log decrease in count of coliforms (7.5 log cfu/g, initially) with 0.15-0.2 mg ozone/L in 3 min, however, there was also 3-4 log reduction with water treatment. Therefore, ozone effect was not evident for the coliform disinfection in lettuce. The treatment (0.3 mg ozone/L for 3 min) also inactivated 4 log *E. coli* O157:H7 in lettuce; initial microbial load was 8.6 log cfu/g. Montecalvo et al. (1998), therefore, found ozone effective against inoculated bacteria on lettuce, which is contrary to what we found in this study. The discrepancy between these two studies may resulted from cell attachment and reactor system. Ogawa et al. (1990) reported that spores of *Bacillus cinerea* on the surface of non-injured tomato fruit were inactivated when exposed to 3.8 mg/L aqueous ozone for 10 min, however, spores placed in injured surface of tomato were not inactivated. This was also true for the treatment with 400 mg/L chlorine solution for 2 min exposures. A similar ozone effect for pear fruit, which was wounded and inoculated with *Penicillium expansum*,
was observed by Spotts and Cervantes (1992). Levels of decay of pears which were treated with 5.5 mg ozone/L for 5 min were similar to those of water treated counterparts. They concluded that ozonated water did not control decay in wound-inoculated pear fruit. Spotts and Cervantes (1992) concluded that ozone and chlorine reach with plant tissue and extracellular biochemical at wound sites and fail to inactivate microbes attached to or embedded in plant tissue.

Hoff (1986) noted microorganisms cultured in the laboratory and those found naturally in the environment are different in resistance to ozone. Cell cultures grown in the laboratory are more easily inactivated. Bacteria that are within particles of feces or other organic matter or that are attached to activated carbon particles are not inactivated as readily as bacteria that are not associated with such particulate matter.

Storage of ozone-treated lettuce. Packaging and refrigeration did not deter microbial growth in ozone-treated lettuce, although ozone treatment decreased the population initially by about half log (Fig. 4.4). Other experiments, however, showed that certain ozone treatments decreased the population of natural flora on lettuce by ~3 log (Fig. 4.7 and 4.12). Therefore, proper ozonation, combined with refrigeration, should improve the shelflife of the treated product. Shewfelt (1986) reported that refrigeration limits growth of spoilage and pathogenic microorganisms and also inhibits respiration and transpiration of plant tissues.

In this study, the count of natural flora on lettuce increase from 4.8-5.3 to 7.6-7.9 log (~2.5 log increase) when the product was held at 7°C for 7 days (Fig. 4.4). According to Splittstoesser (1970), lettuce supported aerobic counts up to $10^7$ cfu/g. Steinbruegge
et al. (1988) demonstrated that *L. monocytogenes* increased by 1 to 3 logs on bite sized lettuce sealed in plastic bags at 5°C and 12°C for 7 days, respectively. Count of naturally occurring *L. monocytogenes* in prepacked ready-to-eat salads increased two-fold when the product was held at 4°C for 4 days (Sizmur and Walker, 1988).

**Effective ozone delivery.**

After it was determined that direct ozone bubbling is more efficient for the lettuce disinfection than ozonated water, 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 will likely accumulate debris and microbial contaminants. Therefore, ozone treatment may become insufficient when applied at conventional vegetable processing environment.

Water washing removed only 0.85 log contaminants from the lettuce (Fig. 4.10). Garg et al. (1990) reported that washing and chlorinated water dips only partially removed the microorganisms that were intrinsic to the vegetables. Water sprays remove many of the surface microorganisms, i.e., those that are not protected by the native mucilaginous material of the plant surface.

**Degree of agitation.** A 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 (Fig. 4.11). Sonication and
stomaching also improved ozone disinfection rate as compared to low-speed stir. Decomposition of ozone is rapid in the water phase of foods that its antimicrobial action may take place mainly at the surface (Kaess and Weidemann, 1968). Most of the microorganisms may not be found in free suspension as discrete particles, specially in food systems. The association of organisms or with suspended matter may hamper the accessibility of ozone to microorganisms.

Ingram and Haines (1949) found that relatively small concentrations (<10 mg/l) of ozone were sufficient to destroy bacteria suspended in water, but higher concentrations were required to kill microbial growth on the surface of agar or meat. The researchers suggested using lower temperature of storage of meat to minimize microbial growth. They also suggested using a rapid atmosphere circulation system to ensure that the ozone is not used up before it reaches contaminants on the meat surface.

Kinetic studies (Chapter 3) showed that microorganisms have no substantial intrinsic resistance to ozone, therefore, it is evident that some of survivors in lettuce treatment had not been in contact with ozone during ozone treatment. Lund (1983) indicated that hypochlorite was ineffective because it reacted with plant tissue components before contacted with bacteria. Adams et al. (1993) also reported that addition of a surface wetting agent, Tween 80, to hypochlorite decreased microbial count by 2 to 4 log, however, this treatment adversely affected the sensory quality.

Inactivation of microbial contaminants on lettuce was enhanced when ozone was bubbled in ozonated water-lettuce mixture (Table 4.7, Fig. 4.10). Farooq et al (1977) found that presence of ozone bubbles, along with ozone residual, was more effective in
inactivating *Candida parapsilosis* and *Mycobacterium fortuitum* than was ozone residues alone. They explained differences in effectiveness in the absence and presence of ozone bubbles by a conceptual model using the film theory. According to this theory, a liquid film forms at the gas-liquid interface, and ozone is more concentrated in the liquid film than in the bulk liquid. This implies that inactivation of microorganisms would be greater when they are in contact with ozone bubbles or within liquid film than with bulk liquid. Therefore, smaller bubbles provide larger surface area and greater inactivation.

Hurst (1993) described a method for sanitizing food products that utilizes ozone containing bubbles. He argued that exposing food products to ozone-air bubbles effectively eliminate bacteria present on the product surfaces. When poultry carcasses were spiked with *S. typhimurium* and immersed in an ozone saturated water bath (35 - 40 mg/L) for 5 min, count of the pathogen was not significantly different from the water control. However, when these spiked carcasses were immersed into the water bath with ozone-air bubbles for 10 min, *Salmonella* count decreased by 2-3 log.

The contacting system, also known as absorption or mixing chamber, is a structure within the treatment process where the ozone in the carrier gas is transferred to the process flow. It is the most important part of any ozonation system and the most influential factor for ozone system failure. White (1972) describes an ideal mixing chamber, which produces large bubble-area to gas-volume ratios, provides enough turbulence to reduce film thickness, and provides large ozone concentration differences between the gas and liquid phases.

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Gaseous ozone treatment. Lettuce samples were flushed with ozone with or without prior vacuuming. Both of these ozone treatment decreased the population by 1 log approximately (Fig. 4.13). Gaseous ozone was used to control microorganisms during the storage of fruits and vegetables. Results from different studies were inconsistent because of differences in methods of applications, ozone measurement, contact time, number and types of contaminants, and product sensitivity to ozone. Some researches found ozone effective against microorganisms in stored produce such as cheese, strawberries, blackberries, banana, and potatoes (Barth et al., 1995; Gibsen et al., 1960; Rice et al., 1982). However, ozone did not controlrots on apples, peaches, cranberries, and green beans (Barger et al., 1948; Brooks and Casllary, 1978; Norton, 1968; Spalding, 1968). Sarig et al. (1996) reported significant reduction of bacteria, fungi, and yeasts on the grapes by 26 min exposure to a gaseous mixture containing 8 mg ozon/L. These authors found significant decrease in fungal decay and increased resistance to post-harvest decay. They concluded that ozone treatments can be considered as a possible substitute for sulphur dioxide fumigation for the control of post-harvest fungal decay. Barth et al. (1995) evaluated blackberries stored in atmosphere containing ozone (0.1 and 0.3 ppm) for 12 days, and reported that ozone suppressed fungal development and peroxidase activity and did not affect the surface color and anthocyanin content in juice. Kute et al. (1995) also reported that storage of strawberries in ozone-containing atmosphere (0.7 ppm ozone) for a week did not decrease the fruits’ level of ascorbic acid.

Mitsuda et al. (1991) noted in their patent that in fresh produce processing, ozonated water is superior to gaseous ozone because the former has greater germicidal
actions and better permeability into foods that the latter does. They used carbon dioxide as an inert carrier gas which improved the penetration power of ozone. The authors gave an example of total destruction of *Bacillus* sp. with ozone-carbon dioxide mixture, while ozone gas only reduced 74% of colonies on the agar.

In conclusion, bubbling ozone gas in water provided the best ozone delivery system for the inactivation 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 tumbling. The inactivation rate of microorganisms is closely related to residual ozone in the treated mixture. For greater inactivation by gaseous ozone, reactor designs need to be improved.

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<table>
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<tr>
<th>Microorganism</th>
<th>Product (positive sample %)</th>
<th>Reference</th>
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<td>Doyle and Schonl (1986)</td>
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</tr>
<tr>
<td>Virus hepatitis A</td>
<td>Lettuce</td>
<td>Rosenthal et al. (1990)</td>
</tr>
<tr>
<td>E. colienteritidis</td>
<td>Salad vegetable</td>
<td>Stocker et al. (1987)</td>
</tr>
</tbody>
</table>

Table 4.1. Foodborne pathogens isolated from minimally processed fresh (MPP) vegetables.
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Product suspected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Bean sprouts</td>
<td>Portnoy et al. (1976)</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Shredded cabbage in coleslaw</td>
<td>Solomon et al. (1990)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Salads or raw vegetables</td>
<td>Merosa et al. (1976)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Alfalfa</td>
<td>Parber et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>Raw vegetables in salads</td>
<td>Ho et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>Salted mushrooms</td>
<td>Juntilla and Branda (1989)</td>
</tr>
<tr>
<td></td>
<td>Shredded cabbage in coleslaw</td>
<td>Schlach et al. (1983)</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Bean sprouts</td>
<td>O'Mahony et al. (1990)</td>
</tr>
<tr>
<td><em>Shigella sonnei</em></td>
<td>Shredded lettuce</td>
<td>Davis et al. (1988)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Cabbage</td>
<td>Swerdlow et al. (1992)</td>
</tr>
<tr>
<td><em>Hepatitis A virus</em></td>
<td>Lettuce</td>
<td>Rosenbluth (1990)</td>
</tr>
</tbody>
</table>

Table 4.1. Pathogens which caused diseases linked to the consumption of raw vegetables.

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<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Decrease (%)</th>
<th>Time (min)</th>
<th>Conc. (mg/l)</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides melaninolyticus</em></td>
<td>100</td>
<td>0.25</td>
<td>3</td>
<td>6</td>
<td>Hays et al. (1963)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>100</td>
<td>1.20</td>
<td>2.3-2.4</td>
<td>7.2</td>
<td>Benzie et al. (1959)</td>
</tr>
<tr>
<td><em>Clostridium butyricum</em> type A</td>
<td>100</td>
<td>0.5</td>
<td>0.5</td>
<td>7</td>
<td>Benzie et al. (1959)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>100</td>
<td>1</td>
<td>0.055</td>
<td>7</td>
<td>Butterfield et al. (1943)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>100</td>
<td>8.5</td>
<td>50</td>
<td>8.4</td>
<td>Coxi (1930)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>99.990</td>
<td>0.1</td>
<td>100</td>
<td>9.5</td>
<td>Lopez (1986)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>100</td>
<td>0.25</td>
<td>5</td>
<td>6</td>
<td>Hays et al. (1963)</td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em></td>
<td>100</td>
<td>3</td>
<td>0.040-0.055</td>
<td>7</td>
<td>Butterfield et al. (1943)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>100</td>
<td>0.5</td>
<td>2.8</td>
<td>7.2</td>
<td>Dybdal (1960)</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>100</td>
<td>2</td>
<td>0.5</td>
<td>7.5</td>
<td>Stuart &amp; Orionio (1964)</td>
</tr>
<tr>
<td><em>Streptococcus pneumonia</em></td>
<td>99.99</td>
<td>5</td>
<td>100</td>
<td>9</td>
<td>Ort &amp; Murray (1989)</td>
</tr>
<tr>
<td><em>Fungi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>100</td>
<td>30-40</td>
<td>100</td>
<td>10-11</td>
<td>Dybdal (1961)</td>
</tr>
<tr>
<td><em>Rhodotorula flocculenta</em></td>
<td>100</td>
<td>5</td>
<td>100</td>
<td>10-11</td>
<td>Dybdal (1961)</td>
</tr>
<tr>
<td><em>Viruses</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poliovirus (Mahoney)</td>
<td>99.9</td>
<td>3</td>
<td>0.21-0.30</td>
<td>7</td>
<td>Clarke &amp; Chang (1959)</td>
</tr>
<tr>
<td>Coxsackie B5</td>
<td>99.9</td>
<td>1</td>
<td>0.21-0.30</td>
<td>7</td>
<td>Clarke &amp; Chang (1959)</td>
</tr>
<tr>
<td>Simian rotavirus</td>
<td>99.99</td>
<td>0.25</td>
<td>0.5</td>
<td>6</td>
<td>Berman et al. (1984)</td>
</tr>
<tr>
<td>S. typhimurium phage strain 144F</td>
<td>100</td>
<td>0.25</td>
<td>25</td>
<td>6.9-8.2</td>
<td>Hays et al. (1959)</td>
</tr>
<tr>
<td><em>Protozoa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endamoeba histolytica cysts</td>
<td>99-100</td>
<td>130</td>
<td>0.08-0.12</td>
<td>7</td>
<td>Clarke &amp; Chang (1959)</td>
</tr>
</tbody>
</table>

Table 4.3. Inactivation of various microorganisms by chlorine.
<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Treatment</th>
<th>Count (log cfu/g)</th>
<th>Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ozone</td>
<td>Control</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>4.5</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>4.5</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td>0.2 mM</td>
<td>4.5</td>
<td>76.6</td>
</tr>
<tr>
<td></td>
<td>0.37 mM</td>
<td>4.5</td>
<td>73.1</td>
</tr>
<tr>
<td>Chlorine</td>
<td>Control</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>4.5</td>
<td>38.3</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>4.5</td>
<td>48.7</td>
</tr>
<tr>
<td></td>
<td>0.17 mM</td>
<td>4.5</td>
<td>22.4</td>
</tr>
</tbody>
</table>

* Data are averages of 2 replications.
* Lettuce was mixed with concentrated water and agitated mildly for 3 min.
* Lettuce was mixed with chlorine solution (1:10, w/v) and agitated mildly for 3 min.
* Chlorine is total available chlorine.

Table 4.4. Inactivation of natural contaminants on fresh lettuce by ozone and chlorine.
<table>
<thead>
<tr>
<th>Ozone treatment*</th>
<th>Chlorine treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7°C</td>
</tr>
<tr>
<td>Control</td>
<td>3.7</td>
</tr>
<tr>
<td>Water</td>
<td>3.5</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>3.3</td>
</tr>
<tr>
<td>0.33 mM</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* Data are averages of 2 replications.
* Lettuce was mixed with ozonated water and agitated mildly for 3 min.
* Lettuce was mixed with chlorine solution (1:10, w/v) and agitated mildly for 3 min.
* Chlorine is total available chlorine.

Table 4.5. Count of natural contaminants on fresh lettuce during the ozone and chlorine treatment at different temperatures.
<table>
<thead>
<tr>
<th>Storage time (day)</th>
<th>Control</th>
<th>Ino+AP</th>
<th>MAP+Ino</th>
<th>Vacuum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Untreated (Log cfu/g)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3</td>
<td>6.8</td>
<td>6.7</td>
<td>7.8</td>
</tr>
<tr>
<td>Ozone treated (Log cfu/g)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>6.4</td>
<td>5.9</td>
<td>7.2</td>
</tr>
<tr>
<td>Survival fraction&lt;sup&gt;c&lt;/sup&gt; (N/N&lt;sub&gt;0&lt;/sub&gt;)</td>
<td>-</td>
<td>0.37</td>
<td>0.17</td>
<td>0.28</td>
</tr>
<tr>
<td>Ozone concentration (ppm)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>5.8</td>
<td>9.7</td>
<td>6.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Control; uninoculated and packaged aerobically; Ino+AP; inoculated and packaged aerobically; MAP+Ino; packaged by modified atmosphere packaging and inoculated; Vacuum; uninoculated and vacuum packaged

<sup>b</sup>One day samples were mixed with water (1:5, w/v) or ozone water in stomach bags for 1 minute.

<sup>c</sup>Survivors fraction = count of ozone treated sample/count of untreated counter part

<sup>d</sup>Ozone concentration was calculated from A<sub>254</sub> using a conversion factor.

Table 4.6. Inactivation of *Pseudomonas fluorescens* on lettuce by ozone.
<table>
<thead>
<tr>
<th>Delivery method</th>
<th>Ozonation method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>Low-speed stir</td>
<td>0.74± 0.06</td>
</tr>
<tr>
<td>Low-speed stir + Sonication</td>
<td>1.02± 0.10</td>
</tr>
<tr>
<td>Low-speed stir + Stomaching</td>
<td>0.84± 0.16</td>
</tr>
<tr>
<td>High-speed stir</td>
<td>1.03± 0.49</td>
</tr>
</tbody>
</table>

*Data are means of population inactivated ± standard error.
**Means with different superscripts in a same row or column are significantly different (P<0.05).

Table 4.7. Decrease in count (4.5-6.3 log cfu/g) of natural contaminants on fresh lettuce by combining ozonation and delivery methods.
Figure 4.1. Experimental setup for the treatment of lettuce with ozone.
Figure 4.2. Treatment of lettuce by different ozonation methods.
Figure 4.3. Methods of delivering ozone for lettuce treatment.
Figure 4.4. Growth of psychrotrophic bacteria on lettuce treated with different disinfectants and stored in modified atmosphere packaging at 7°C. The concentration of chlorine and ozone was 0.2 mM in aqueous solutions.
Figure 4.5. Counts of natural contaminants on fresh lettuce after treatment with chlorine at 25°C.
Figure 4.5. Counts of natural contaminants on fresh lettuce after treatment with 1.3 mM ozone at 25° C.
Figure 4.7. Inactivation of mesophilic natural contaminants (1.6x10^4 cfu/ml, initially) on lettuce by oxygen (control) and bubbling ozone (1.5 mM) treatment. N₀: count of untreated sample, N: count of treated sample.
Figure 4.8. Inactivation of psychrotrophic natural contaminants (1.4x10^5 cfu/ml, initially) on lettuce by oxygen (control) and bubbling ozone (1.3 mM) treatment. No; count of untreated sample, N; count of treated sample.
Figure 4.9. Inactivation of *P. fluorescens* (2.6x10^5 - 4.9x10^6 cfu/ml, initially) on lettuce by ozone. \textit{Ino+AP}; Lettuce inoculated and packaged in atmospheric packaging; \textit{MAP+10o}; Lettuce packaged under modified atmosphere and inoculated after packaging. N0; count of untreated sample, N; count of treated sample.
Figure 4.10. Inactivation of natural contaminants (2.8×10^6 - 2.1×10^6 cfu/ml, initially) on fresh lettuce by different ozonation. Means and standard errors were shown. Different letter indicates significant difference at p<0.01. N₀: count of untreated sample, N₁: count of treated sample.
Figure 1. Incubation of natural communities (2 x 10^4) on fresh old sludge, plus different agitation methods: 1) Low-speed stir, 2) Low-speed stir plus vibration, 3) Shaker, 4) High-speed stir. Means and standard errors are shown. Different letters indicate significant differences at p<0.05.
Figure 4.12. Inactivation of natural contaminants (5.5 × 10^4 cfu/ml, initially) on lettuce by ozone at different stir speed, and associated changes in residual ozone. No. count of untreated sample, N2; count of treated sample.
Figure 4. Inactivation of natural contaminants (2.0x10^5 cfu/ml, initially) on lettuce by different ozone delivery methods in gaseous ozone treatment. 1; Water washed and flushed with ozone, 2; Unwashed and flushed with ozone, 3; unwashed, vacuumed, and flushed with ozone. No; count of untreated sample, N; count of treated sample.
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