Inactivation Kinetics of Foodborne Spoilage and Pathogenic Bacteria by Ozone

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

Key Words: ozone, inactivation, kinetics

Introduction

INACTIVATION OF BACTERIA BY OZONE is commonly studied in batch systems, and single endpoint determinations of viability are reported (Edelstein and others 1982; Farooq and others 1977; Finch and others 1988; Hunt and Marinas 1997; Katzenelson and others 1974). Results on relative resistance of food-borne spoilage and pathogenic bacteria to ozone are inconclusive (Boyce and others 1981; Broadwater and others 1973; Farooq and Akhlaque 1983; Restaino and others 1995). Additionally, minimal information is currently available about inactivation of emerging food-borne pathogens (for example, *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 food-borne microorganisms, (b) develop a method to study kinetics of microbial inactivation by ozone, and (c) use the kinetics data to predict the inactivation of microorganisms over a broad range of ozone concentrations.

Results

Inactivation studies

Batch reaction. Range of ozone concentrations varied, for practical reasons, when different microorganisms were tested. Sensitivities of treated bacteria were compared at similar ozone concentrations or by using dose-response plots. Sensitivities of *E. coli* O157:H7 and *Pseudomonas fluorescens* to ozone were slightly different. Ozone at 0.2 ppm inactivated 0.9 log of *P. fluorescens* in 30 s, whereas 1.2 ppm decreased the population by 5 log in a similar treatment time (Fig. 1a). When *E. coli* O157:H7 was treated with 0.3 and 1.0 ppm ozone, the count decreased 1.3 and 3.8 log, respectively, in 30 s (Fig. 1b).

Counts of *Leuconostoc mesenteroides* decreased by 1.3 and 3.3 logs when initial ozone concentrations were 0.3 and 1.5 ppm, respectively (Fig. 1c). Ozone at about 4 ppm killed about 7 log *L. mesenteroides* CFU/mL. Ozone at 0.4 and 0.8 ppm, initially, inactivated 4.6 and 5.7 log *L. monocytogenes* CFU/mL (Fig. 1d). Therefore, microorganisms tested in this study showed similar inactivation kinetics. Most inactivation occurred during the

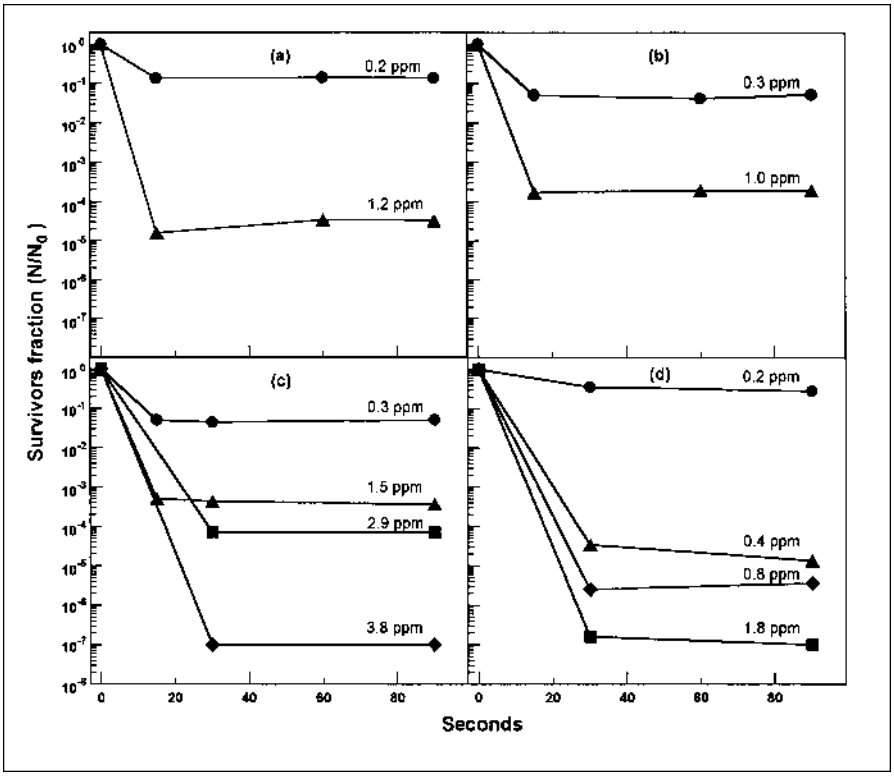

Fig. 1—Survivors plots for the inactivation of (a) *P. fluorescens*; (b) *E. coli* O157:H7; (c) *Leu. mesenteroides*; (d) *L. monocytogenes* (1.3×10^8 – 2.5×10^8 cfu/mL, initially) by ozone in the batch reactor at pH 5.9 and 25 °C. N_0: count of untreated sample, N: count of treated sample.

first 15 or 30 s of the treatment, and counts remained unchanged when the mixture was held for up to 6 min (results for the first 90 s only are shown in Fig. 1).

Correlation between amounts of ozone remaining in the reaction mixture (residual ozone) and degree of inactivation was studied. A cell suspension of *L. mesenteroides* (about 10^7 CFU/mL) was mixed with ozonated water to contain 0.3 to 2.1 ppm ozone, initially. The mixture was sampled to determine counts of survivors and the residual ozone (Fig. 2a). When the initial ozone concentrations were 0.3, 1.1, and 2.1 ppm, residual ozone concentrations after 30 s, were 0.0, 0.5, and 0.9 ppm, respectively. Bacterial counts after 30 s of exposure were 2.2, < 1 and <1 log CFU/mL, respectively (data not shown). Therefore, inactivation of bacteria by ozone is a rapid process; this process continues until either survivors or residual ozone becomes undetectable. Estimated ozone demand of 10^7 *L. mesenteroides* CFU/mL is 0.83 ppm. Therefore, about 10^9 molecule of ozone were used to inactivate each cell.

Inactivation of *L. mesenteroides* by 1.3 ppm ozone was investigated when initial count varied (Fig. 2b). When initial inoculum sizes were 6.8, 7.1, 7.4, and 7.7 log CFU/mL, ozone concentration decreased by 62%, 58%, 83%, and 100%, respectively, during 15 s of treatment. A large decrease in residual ozone was observed initially, and the rate of decrease diminished when the mixture was held for up to 3 min (Fig. 2b). A similar trend of inactivation kinetics was observed, that is, rapid decrease in count initially and minimal changes later (data not shown). Therefore, effectiveness of ozone varied considerably with inoculum sizes. Inactivation by 1.3 ppm ozone was less than 1 log for the largest inoculum (7.7 log CFU/mL), but it was > 6 log when an inoculum half the size of the former (7.4 log CFU/mL) was used. Therefore, ratio between amounts of treated cells and added ozone should be considered carefully for maximum effectiveness of ozone.

In the batch reaction system, microorganisms are inactivated rapidly (< 30 s), and thus determination of inactivation kinetics is technically difficult (Fig. 1). 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. 3a). Data were linear at 2 ranges of ozone concentrations. *P. fluorescens* PI values changed considerably with ozone concentration up to about 1 ppm. But theses values increased only moderately at concentrations > 1 ppm. Similar inactivation kinetics were observed when *E. coli* O157:H7, *L. mesenteroides,* and *L. monocytogenes* were tested (Fig. 3b to 3d).

PI-value at 1 ppm ozone was determined from equations describing the dose-response plots; these values are 4.6, 2.7, 3.5, and 7.5 log for *P. fluorescens*, *E. coli* O157:H7, *L. mesenteroides*, and *L. monocytogenes*, respectively. *E. coli* O157:H7 was the most resistant, while *L. monocytogenes* was the least resistant against ozone inactivation. Increase in PI values with increase in initial ozone concentration (that is, slope of the first segment of the dose-response plots) was greatest for *L. monocytogenes*. In conclusion, ozone concentration and cell inactivation are linearly-related over 2 ranges of ozone concentrations.

Continuous reactions

Reactor I. Unlike the batch system, this reactor permitted measuring bacterial inactivation after short periods (0 to 20 s) of exposure to ozone (Fig. 4). Exposure to 0.1-0.7 ppm ozone caused 0.7 to 7.0 log decrease in count, depending on the microorganisms. Most of the decrease in count occurred during the first 5 s of the treatment (Fig. 5). Count of *L. monocytogenes* decreased gradually as contact time

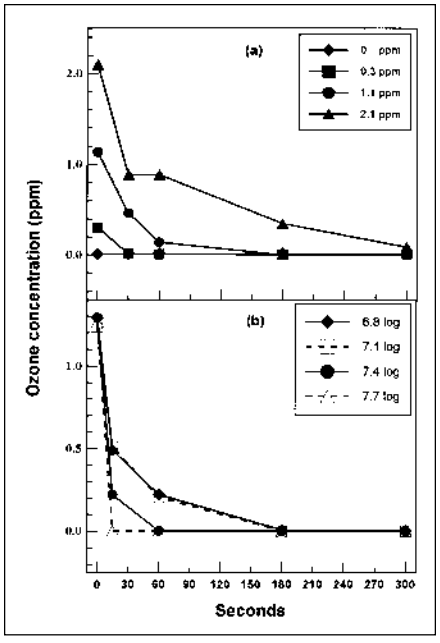

Fig. 2—Changes of residual ozone concentration when variable initial ozone and *Leu. mesentreroides* cell concentrations (a) ~10^7 cfu/mL and variable initial ozone; (b) variable inoculum size and initial ozone concentration of 1.3 ppm.

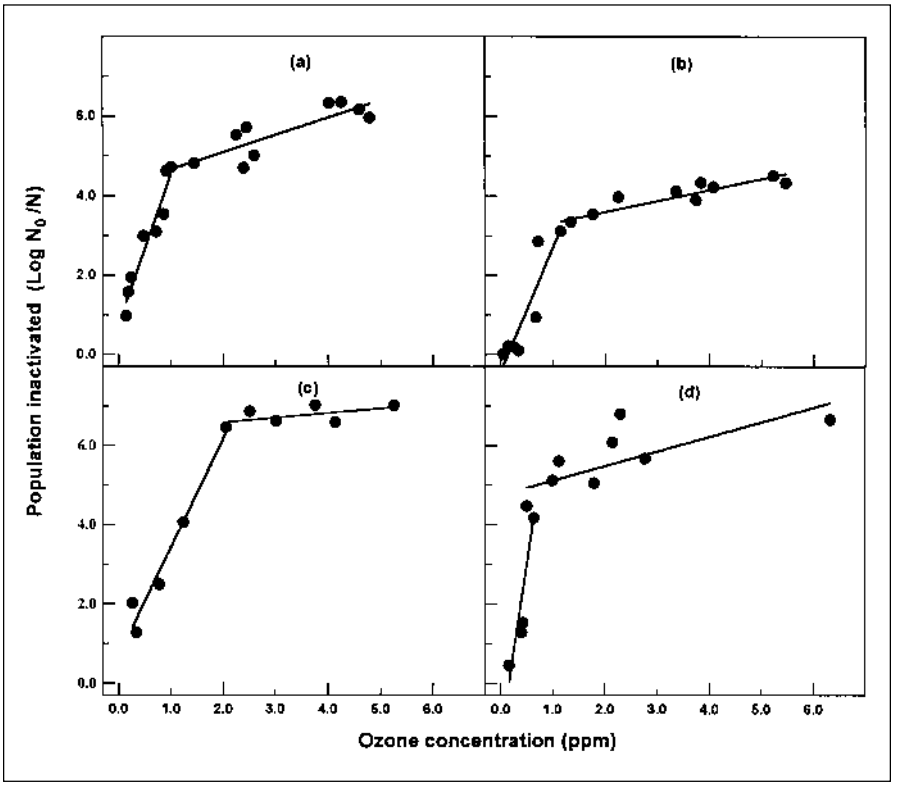

Fig. 3—Dose-response plots for the inactivation of (a) *P. fluorescens*; (b) *E. coli* O157:H7; (c) *Leu. mesenteroides*; (d) *L. monocytogenes* (1.3×10^8 – 2.5×10^8 CFU/mL, initially) by ozone in the reactor at pH 5.9 and 25 °C. N_o: count of untreated sample, N: count of treated sample.

increased (Fig. 5d); this kinetic pattern is more distinctive than that seen in other bacteria. Resistance of *Listeria* against ozone was the least among the tested microorganisms; about 0.4 ppm ozone inactivated about 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.

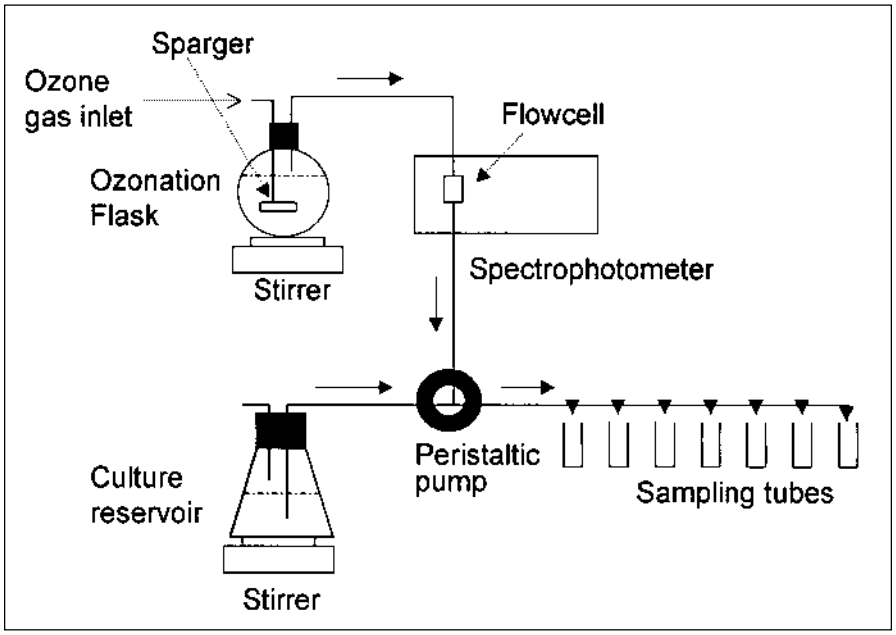

Fig. 4—Continuous reactor I

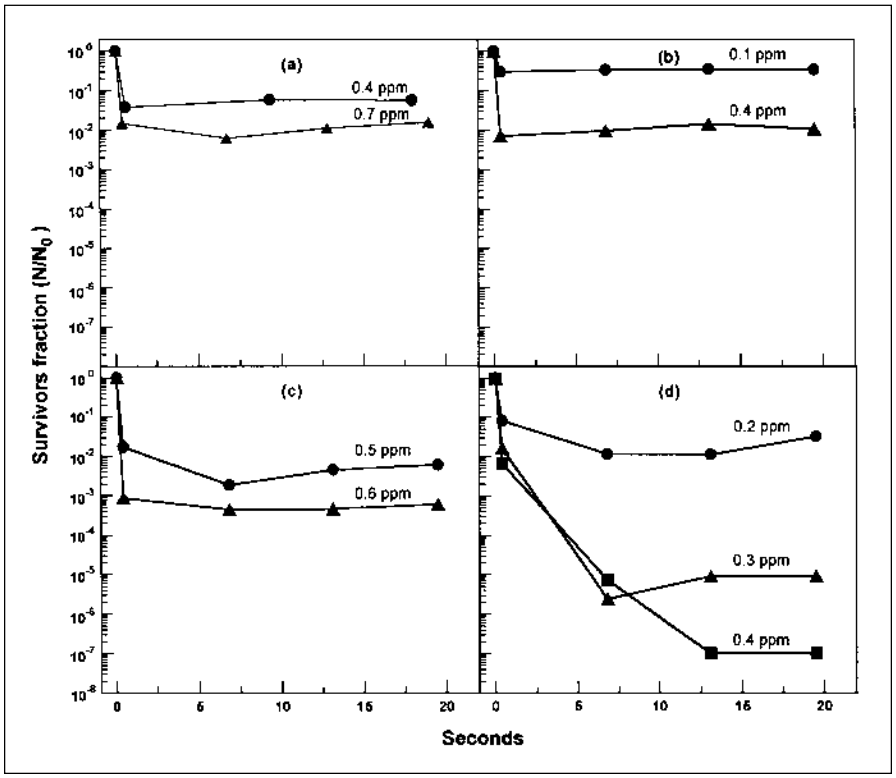

Fig. 5 (above) —Survivors plots for the inactivation of (a) *P. fluorescens*; (b) *E. coli* O157:H7; (c) *Leu. mesenteroides*; (d) *L. monocytogenes* (9.6 x 10^7 - 4.3 x 10^8 CFU/mL, initially) by ozone in the continuous reactor I at pH 5.9 and 25°C. N_o: count of untreated sample, N: count of treated sample.

Reactor II. Inactivation kinetics were different with this reactor than with the previously tested systems. Survivors and dose-response plots were linear over a broader range of contact times and ozone concentrations, respectively. Inactivation of microorganisms by ozone also followed a concave downward curve at all ozone concentrations tested (Fig. 6).

D-values were calculated from initial slopes of survivors plots, that is, during the first 5.4 to 8.5 s of exposure to ozone. D-values at different ozone concentrations were plotted against ozone dosage to construct log-log dose-response plots (Fig. 7). Inactivation of some bacteria (for example, *P. fluorescens*) by ozone gave a scattered dose-response plot, but the relationship between D-value and ozone dosage was clearly linear. For the comparison of ozone resistance, D-values at 1 ppm ozone treatment for the tested microorganisms were calculated. They are 4.6 s for *P. fluorescens*, 6.2 s for *E. coli* O157:H7, 4.4 s for *L. mesenteroides*, and 3.3 s for *L. monocytogenes*. *E. coli* O157:H7 was the most resistant against ozone, while *L. monocytogenes* was the least resistant, which agrees with the results obtained from the batch and the continuous reactor I.

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

Discussion

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

required for 99% kill.

Residual ozone was determined in order to reveal the mechanism involved in the kinetics in the batch reaction (Fig. 2). Ozone decomposed very quickly while it reacted with microorganisms. When the microbial load was large relative to the amount of added ozone, residual ozone was not measurable, but bacterial survivors were detected. When the ratio of ozone to microbial load was large, residual ozone was detected, but the bacterial population was eliminated. Therefore, the extent of inactivation depends on the ozone demand of the added microbial load. Gomella (1972) stated that evidence of a trace ozone residual is an acceptable sign of complete disinfection in water treatment. However, Sommerville and Rempel (1972) reported the presence of coliforms in water containing 0.1 mg/L ozone residual. Farooq and others (1977) observed the degree of inactivation was profoundly affected by the initial organism population. These authors detected 4-log reduction when the initial density of *Candida parapsilosis* was $1.4x10^5$ CFU/mL, but observed no inactivation when the initial density was $1.6x10^7$ CFU/mL.

The inactivation process is an interaction between ozone and the microorganism, analogous to a chemical reaction that follows the course of a first-order reaction (Shechter 1973). In an ozone demand-free reactor system, the only source of ozone demand is the seeded microorganisms. Ozone reacts with cells in the treated water, liberating new molecules capable of reacting with ozone. Therefore, ozone decreases chemical oxygen demand (COD), whereas it increases 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 and others (1988) found that 10^6 *E. coli* cells demanded 0.06 mg/L ozone after lysis and attributed the second phase of inactivation to this ozone demand.

Relative ozone resistance

Batch reactor. In batch reactions, *E. coli* O157:H7 was more resistant to ozone inactivation, and *L. monocytogenes* was less resistant than other tested microorganisms (Fig. 1). Variation in resistance to ozone among microorganisms was reported earlier. Baumann and Ludwig (1962) compared chlorine resistance of different bacteria and viruses and reported that *E. coli* at pH 7 is the most sensitive and *Bacillus anthracis* is the most resistant. Zhao and Cranston (1995) observed a 5 log decrease for *Staphylococcus aureus*, *B. cereus, E. coli,* and *Salmonella* in 10 to 20 min when they were sparged in the water with 6.7 mg/L ozone at the flow rate of 6 L/min. They also reported that *S. aureus* and *B. cereus* exhibited greater tolerance to ozone than did *E. coli* and *Salmonella* spp.

Inactivation data were fitted to a dose-response model having 2 segments (Fig. 3). According to Finch and others (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 tube, but ozone was quickly depleted during the holding period (Fig. 5). Although this design maximizes bactericidal action of ozone and minimizes ozone depletion by intracellular components, data were only marginally better in describing inactivation kinetics than were the data from the batch system (Fig. 5).

The reaction system was modified to permit continuous exposure of bacteria to constant concentration of ozone during the treatment. Inactivation data were better described by first-order kinetics when using reactor II than the previously tested systems (Fig. 6). 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. 7).

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

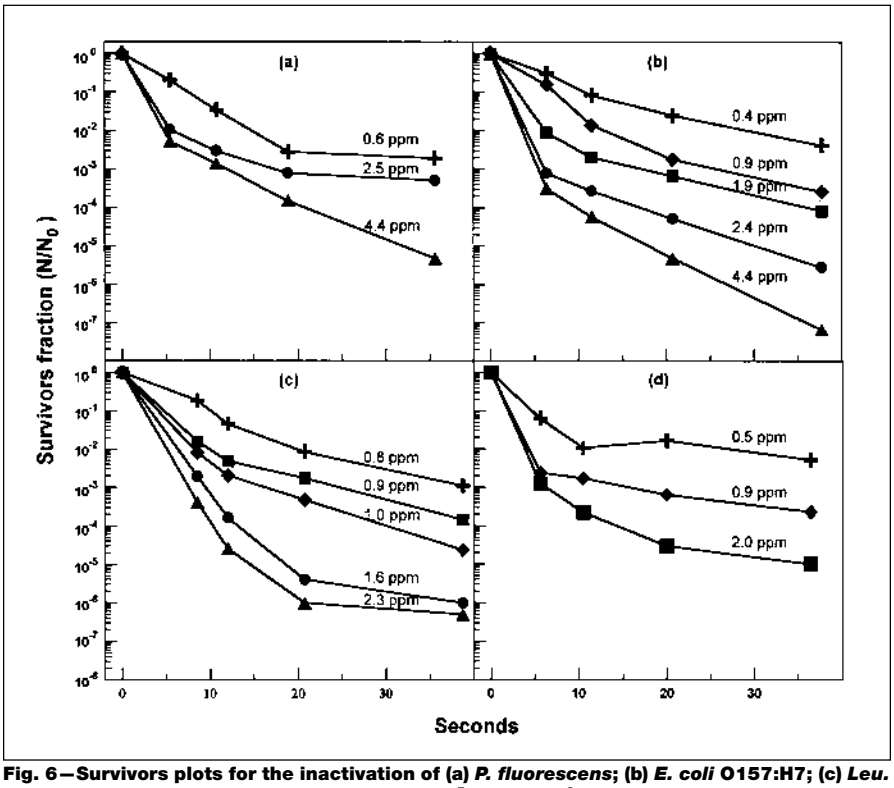

Fig. 6—Survivors plots for the inactivation of (a) *P. fluorescens*; (b) *E. coli* O157:H7; (c) *Leu. mesenteroides*; (d) *L. monocytogenes* (6.6×10^7 - 2.2×10^8 cfu/mL, initially) by ozone in the continuous reactor II at pH 5.9 and 25°C. N_0: count of untreated sample, N: count of treated sample.

creased > 5 log immediately, but only a decrease of 3 log was observed for *S. aureus*, *E. faecalis*, and *B. cereus*. Most bacteria showed biphasic death curves. Restaino and others (1995) concluded that the gram-negative bacteria were substantially more sensitive to ozonated water than the gram-positive bacteria, which is inconsistent with the findings of this study.

Hunt and 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 5 to 7 log units of inactivation. They related the tailing of survivor plots to the presence of bacterial clumps in the reaction mixture. In the experiments performed with the tubular reactors, Hunt and Marinas (1997) found that the concentration of dissolved ozone decreased as it reacted with the treated microorganisms. Their data showed 2 phases of kinetics. In addition, from their inactivation study with or without a radical scavenger, tert-butanol, they concluded that molecular ozone rather than free radicals was primarily responsible for inactivation in the range of experimental conditions examined.

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

Ozone disinfection had 2 distinct stages: an initial rapid decline in the first stage followed by a slower decline in the second stage (Finch and others 1988; Katzenelson and others 1974). Cellular debris from the damaged or lysed organisms shielded the surviving *E. coli* from the effects of ozone (Finch and others 1988). These authors calculated that 3 x 10^8 molecules of ozone were used for each bacterium, however, a 7 log unit reduction in *E. coli* required 45 times more ozone than the predicted value. Consequently, Finch and others (1988) found a dose-response relationship that has a "tail."

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

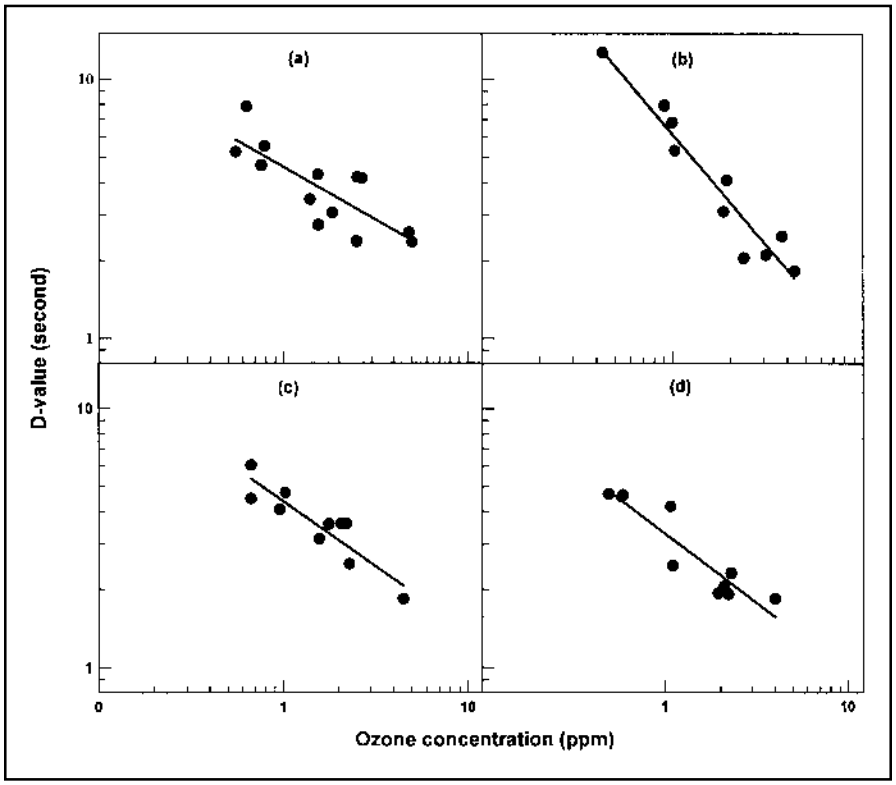

Fig. 7—Log-log dose-response plots for the inactivation of (a) *P. fluorescens*; (b) *E. coli* O157:H7; (c) *L. mesenteroides*; (d) *L. monocytogenes* (1.3 x 10^8 - 2.5 x 10^8 CFU/mL, initially) by ozone in the continuous reactor II at pH 5.9 and 25°C. N_o: count of untreated sample, N: count of treated sample.

Conclusions

OZONE INACTIVATES BACTERIA RAPIDLY, and no viable cells are detectable when residual ozone is present in the reaction mixture. Ozone exerts its action within a few s, and therefore inactivation kinetics are not measurable in the batch and the continuous reactor (type I) modes. A continuous ozone reactor II was developed to ensure a uniform exposure of bacterial cells to ozone and constant concentration of ozone during the treatment. When using this system, inactivation kinetics that are consistent with general disinfection patterns were observed. Therefore, inactivation of microorganisms in a fluid system by aqueous ozone follows patterns that depend on the method of application. Resistance of tested bacteria to ozone followed this descending order: *E. coli* O157:H7, *P. fluorescens, L. mesenteroides*, and *L. monocytogenes*.

Materials and Methods

Preparation of inoculum

P. fluorescens ATCC 17386, *E. coli* O157:H7 ATCC 35150, *L. mesenteroides* subsp. *mesenteroides* ATCC 14935, *L. monocytogenes* Scott A were used in this study. Stock cultures of these bacteria were stored at -20°C in suitable broth media supplemented with 10% (v/v) glycerol. Inoculum of *L. monocytogenes* Scott A was prepared as described by Lou and Yousef (1996). The stock culture of *L. monocytogenes* was inoculated into trypticase soy broth (BBL, Cockeysville,

Md., U.S.A.) supplemented with 0.6% Bacto yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.) (TSBYE) and the mixture was incubated at 35°C for 24 h; this was followed by 2 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, Del., U.S.A.) and washed twice in 0.1 M phosphate buffer solution (pH 7) to a final concentration of 1-3 x 10^9 CFU/mL. Similar protocols were used to prepare inocula of the other bacteria, but growth media and incubation condition were different. *E. coli* O157:H7 was subcultured twice in trypticase soy broth (TSB) and incubated at 35°C for 24 h. *P. fluorescens* was grown in nutrient broth (Difco) and incubated at 26°C for 24 h. *L. mesenteroides* was cultured in Lactobacilli MRS broth (Difco) and incubated at 26°C for 24 h. Inoculum sizes of different bacteria were estimated by measuring Absorbance at 600 nm (A_{600}) and calculating approximate counts from standard curves for absorbance in contrast to bacterial count.

Ozone demand-free glassware and water

All glassware was washed with a mild detergent, thoroughly rinsed with hot tap water and deionized water, autoclaved, and dried. Deionized water was obtained from a Milli-Q system (Model OM-140, Millipore). Ozone demand-free water was prepared by ozonating deionized water (Fig. 8). 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 and others 1990).

Ozone production

Ozone (1.1 mM; about 2.5%, v/v; about 3.7%, w/w) was produced from purified, extra dry oxygen by an ozone generator (U.S. Filter/Polymetrics T-816, San Jose, Calif., U.S.A.). The amount of ozone produced by the generator and that available for the treatment were determined as indicated later. All experimental work with ozone gas and ozonated water was done in a chemical hood. Excess ozone was neutralized by diverting the gas stream into a reservoir containing 2% Potassium Iodine solution. Protective cloth, gloves, and masks were worn while running the experiments.

Measurement of ozone concentration

Ozone concentration was determined by ultraviolet (UV) spectrophotometry and the indigo method (Bader and Hoigne 1981). In 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, N.Y., U.S.A.). Ozone concentration was also determined by decolorization of indigo trisulfonate (Aldrich Chemical Co., Inc., Milwaukee, Wisc., U.S.A.) and measurements of changes in color at 600 nm and pH < 4. The indigo molecule contains only 1 carbon-carbon double bond that reacts with ozone at a very high reaction rate constant (Bader and Hoigne 1981). For the measurement of residual ozone during the reaction, water containing cells was used as a blank before monitoring the absorbance of the reaction mixture.

Preparation of aqueous ozone

Ozonated water was obtained by bubbling ozone about 2.5% (v/v, in oxygen carrier gas) into a round-bottom flask containing about 1000 mL sterile deionized water at 25°C (Fig. 8). The flow rate of ozone into the flask was controlled by a peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, Ill., U.S.A.). A stainless-steel sparger with 10 µm pore size (Solvent Inlet Filter, Fisher Scientific, Fair Lawn, N.J, U.S.A.) was used for bubbling ozone in the water. The ozonated water was circulated by a peristaltic pump (Cole-Parmer) through Norprene tubing (Cole-parmer) to the spectrophotometer's flowcell (0.6 mL capacity, Fisher Scientific, Fair Lawn, N.J, U.S.A.) with a light path of 1 cm. The spectrophotometer was used to continuously measure ozone absorbance at 258 nm. The rate of ozone flow was predetermined to achieve and maintain the desired equilibrium ozone concentration. Equilibrium was attained when absorbance at 258 nm remained relatively constant.

Inactivation studies

A batch and 2 continuous reaction systems were setup to study the inactivation of selected microorganisms by ozone. Because of reactivity of ozone, only glass containers and tubes and Norprene tubing were used. The pH and temperature were kept constant during the treatment, but ozone concentration (or dosage) and contact time were varied.

Batch reaction system. Batch reaction simply involves mixing a predetermined volume of ozonated water and cell suspension in a container and neutralizing the mixture after it was held for a given time (Fig. 9). Ozonated water (95 mL) was transferred to a reaction vessel, and ozone concentration was determined by absorbance measurement. The vessel was inoculated with 5 mL cell suspension (in 0.05 M phosphate buffer) to attain a count of about 10^8 CFU/mL. Vessel contents were stirred using a

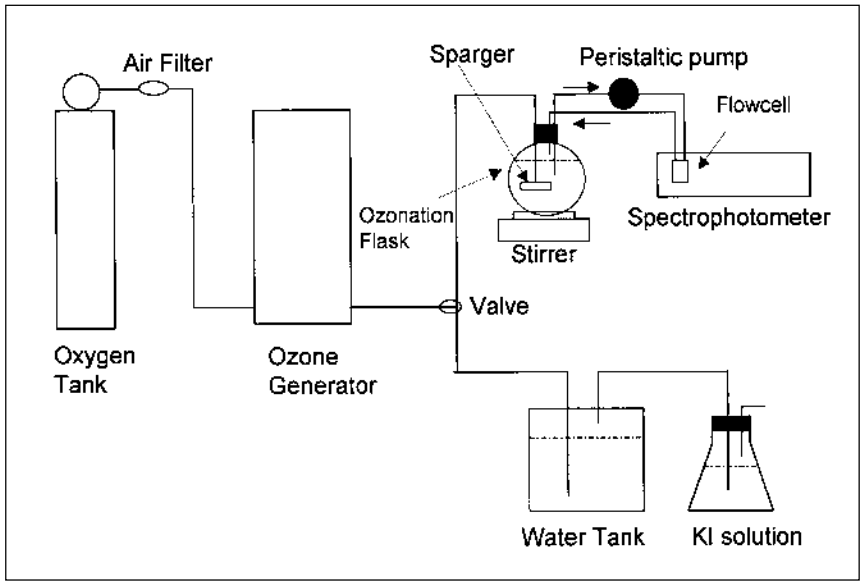

Fig. 8—Production of ozonated water

Teflon-coated magnetic stirrer bar at 100 rpm. Samples (5 mL each) of the reaction mixture were taken at intervals, and each was mixed immediately with 0.5 mL neutralizing solution (0.005 M sodium thiosulfate) 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 s before the ozone neutralizer (0.5 mL) was added to stop the reaction. In this case, samples of ozonated water were taken immediately before adding cell suspensions to determine ozone concentration by the indigo method.

Continuous reaction system. Two continuous reactors (designated I and II) were setup as follows.

Reactor I. The reactor (Fig. 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 2 identical pump heads. These 2 streams were mixed in a y-shaped Norprene tubing (Internal dia; 1.6 mm), and the mixture was carried through the tubing, which has 7 sampling ports (3-way valves) at different lengths. Samples of equal size were collected from ports and mixed immediately with thiosulfate solution (1.24 g/L added at 10% of the sample volume) to neutralize residual ozone. Contact time(s) was calculated as follows: {resident volume between the mixing point and a sampling port / Flow rate (mL/min)} × 60. The calculated contact time ranged from 0.4 to 37.8 s. Survivors were counted in collected samples.

Reactor II. A membrane filter (pore size, 0.45 µm; dia, 25 mm; composition, mixed cellulose acetate and nitrate (HAWP, Millipore) was mounted on the fritted glass base of a glass filtration unit (Glass Microanalysis, Millipore, Burlington, Mass., U.S.A.), and the unit was assembled. The funnel functions as a reservoir for the ozonated water (Fig. 10). Cell suspension (1 to 2 mL, about 10^8 CFU/mL) and subsequently ozonated water (1 to 10 mL) were drawn through the filter at a constant rate (22 mL/min) using a peristaltic pump (Cole-Parmer). Contact time(s) was calculated as follows: {Volume of applied ozonated water / Flow rate (mL/min)} x 60. Dosage was varied by using variable volume (1 to 10 mL) of ozonated water or by applying equal volumes of water that contained different concentrations of ozone. The filter with the treated cells was transferred into peptone water for ozone neutralization

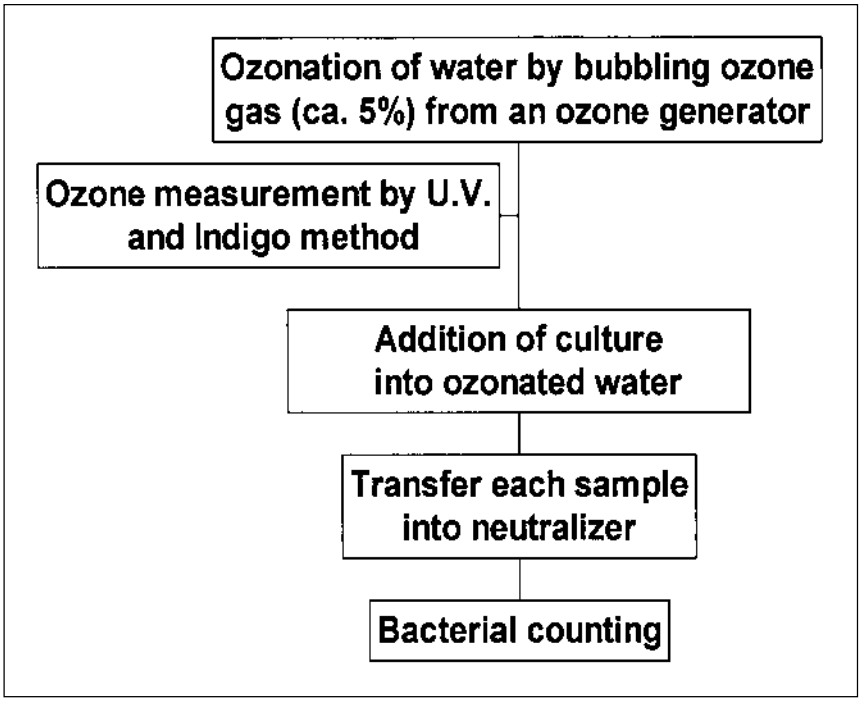

Fig. 9—Kinetic study by the batch reaction system

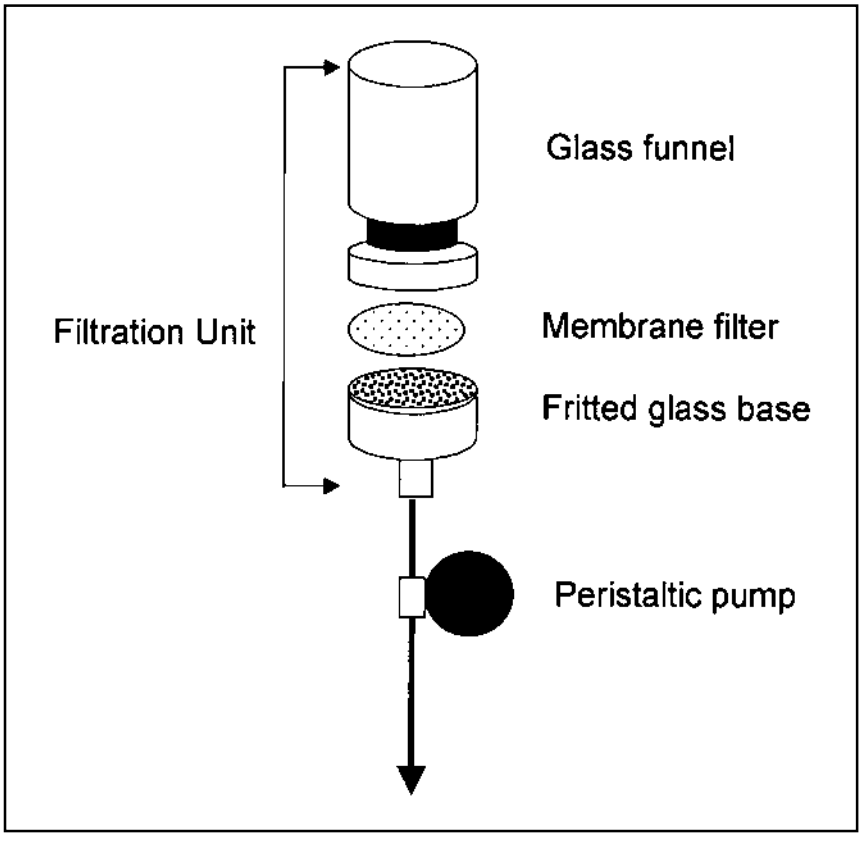

Fig. 10—Continuous reactor II

and cell detachment by vigorous agitation. Dilutions of samples and plate counting were performed.

Neutralization

Sodium thiosulfate (0.005 M) neutralizer stock solution was prepared by dissolving 1.24 g of $Na_2S_2O_3.5H_2O$ (Sigma Chemical Co., St. Louis, Mo., U.S.A.) in 1 L of freshly autoclaved distilled water (Rand and others 1975). The amount of sodium thiosulfate solution was varied depending on the estimated ozone concentration in the solution being neutralized. This neutralizer does not have an interfering absorbance at 258 nm, nor an adverse effect on treated microorganisms (Kim and others 1999a)

Microbiological tests

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

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