UNIVERSITY OF ALBERTA

OZONE INACTIVATION OF *LISTERIA INNOCUA* IN DEMAND-FREE PHOSPHATE BUFFER

BY

DALE HARVEY ADAMS ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Ozone Inactivation of *Listeria innocua* in Demand-Free Phosphate Buffer** submitted by **Dale Harvey Adams** in partial fulfillment of the requirements for the degree of **Master of Science** in **Environmental Engineering**.

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ABSTRACT

A study of the effect of ozone on *Listeria innocua* suspended in demand free 0.1 M phosphate buffer (pH 6.9) was undertaken. The organism was propagated in tryptic soy broth yeast extract (TSBYE) and incubated at room temperature. The culture was centrifuged. The resulting pellet was washed twice and resuspended in phosphate buffer. Stock organism solution was added to reactor vessels to obtain an average initial concentration of approximately 7×10^5 CFU/mL.

A series of bench scale experiments using ozone demand free reaction vessels was conducted at temperatures of 22 °C and 3 °C. After control samples were removed from the reaction vessel, stock ozone solution was added. UV spectrophotometry was used to measure ozone stock solution concentrations as well as initial and final ozone residuals in the reaction vessel. At the end of the contact time, excess ozone was quenched with 1 M sodium formate.

The inactivation kinetics of *L. innocua* by ozone was described by a nonlinear Hom model. Adequacy of the model was checked by lack-of-fit tests and examination of the residuals. Precision of the parameter estimates was obtained from examination of joint confidence regions. Very similar Hom model parameter estimates were obtained for data sets at both temperatures.

L. innocua appeared to be equally sensitive to ozone disinfection at 3° and 22 °C. Comparison of the inactivation data with previous research indicates that *L. innocua* is extremely sensitive to ozone disinfection. A short contact time (1 minute) and low average ozone dose (0.1 mg/L) resulted in a log survival ratio of 4.8 at 22 °C. Similar results were obtained at 3 °C.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

1.1. Problem Definition

Listeria monocytogenes is an ubiquitous microorganism that can cause listeriosis in man and in many species of animals(Jones and Seeliger 1992). Listeriosis is a term that covers a wide variety of clinical manifestations ranging from mild influenza-like symptoms to meningitis. The populations most at risk include immunocompromised individuals, the elderly, pregnant women, and newborns (Lovett and Twedt 1988). Infection of the fetus can lead to abortion, stillbirth, or delivery of an acutely ill infant.

Foodborne transmission of *L. monocytogenes* has been implicated in human cases of listeriosis that have been traced to consumption of cooked chilled chicken, coleslaw, raw vegetables, milk, Mexican-style cheese and Swiss cheese (Johnson et al. 1990; Jones and Seeliger 1992).

L. monocytogenes has been isolated from many types of mammals, birds, fish, seafood, vegetables, soils, water, raw milk and raw meat. The percentage of retail poultry meat samples that are positive for *L. monocytogenes* ranges from 10-66%. Although the number of cases of listeriosis traced to raw meat products is quite low at the present time, the high incidence rate of *L. monocytogenes* on raw poultry products indicates that they are suspect as a source of infection.

Since raw poultry meat is also a source of several other pathogenic organisms, a great deal of effort has been invested in finding a potent disinfectant that will destroy the pathogenic bacteria but not have an adverse effect on the meat product. To date a successful candidate has not been found.

Ozone (O_3) is a strong but unstable oxidant. Disinfection studies using ozone and bacteria suspended in laboratory media have demonstrated that ozone is one of the most potent disinfectants. Research using ozone as a disinfectant during immersion chilling of poultry has indicated that ozone was effective in destroying bacteria in chiller water (Adams 1990; Sheldon and Brown 1986). However, reduction of numbers of bacteria on poultry meat surfaces was not as large as might be expected given the potency of ozone as a disinfectant. It appears that insufficient attention in the design of these experiments was given to ensuring that an adequate ozone residual concentration was maintained at the liquid/gas/solid interface.

The work carried out for this thesis was the first stage in a research project looking at the use of ozone as a disinfectant for poultry meat. The goal of the first stage was to characterize how a microorganism (*L. monocytogenes*) common to raw poultry meat would respond to ozone disinfection when suspended in phosphate buffer at room temperature (22 °C) and at immersion chiller temperatures (4 °C) by using a very closely related nonpathogenic strain - *L. innocua*. The inactivation data were used to determine the kinetic parameters of the Hom model allowing comparisons to be made with the results from other ozone disinfection studies that have used the same protocol.

1.2. Objectives

This study had three objectives:

1. to conduct a series of experiments at 22 °C using a range of ozone doses and contact times to inactivate *L. innocua*.
2. to conduct a series of experiments at 3 °C also using a range of ozone doses and contact times to inactivate *L. innocua*.
3. to determine the Hom model parameters (k, m, n) for the inactivation data using the method of nonlinear least squares.

1.3. Scope

The main thrust of the study was to gain an understanding of how *L. innocua* responds to disinfection by ozone in demand free phosphate buffer at room temperature and at 3 °C which is a typical temperature at which poultry chillers are operated. To achieve this goal, a literature survey was undertaken to summarize briefly the health impact caused by listeriosis; to examine the biochemical characteristics of *L. innocua* and *L. monocytogenes* to see if *L. innocua* is a suitable surrogate for *L. monocytogenes* in disinfection experiments; to examine the incidence rates of *L. innocua* and *L. monocytogenes* in the poultry industry; to review ozone chemistry; to critique experiments using ozone to inactivate microorganisms and gain an appreciation of their relative resistance to ozone; and to review the experience gained so far in using ozone as a disinfectant during the chilling of poultry meat.

The research consisted of two segments:

1. experiments were performed using a range of ozone doses and contact times to determine the level of inactivation of *L. innocua* for each combination.

2. analysis of data using nonlinear least squares allowed determination of Hom model parameters (k, m, n) for inactivation of *L. innocua* at 22 °C. The Hom model parameters were also determined for inactivation or the organism at 3 °C.

2. Literature Review

2.1. Description of Listeriosis

The disease caused by *L. monocytogenes* is called listeriosis and it can take many different forms. Pregnant women, their off-spring, and immunocompromised individuals are most susceptible to listeriosis (Bailey et al. 1989). Older people are at greater risk as was shown in a 1986 study where 84% of the patients with listeriosis were over 50 years of age (Gellin et al. 1991). Listeriosis in pregnant women may cause stillbirth, abortion or neonatal sepsis (Lovett 1989). Some symptoms of listeriosis of the newborn include respiratory distress, heart failure, vomiting, and convulsions (Marth 1988). Meningitis and meningoencephalitis caused by *L. monocytogenes* can develop in newborns and in older people with a fatality rate of approximately 70% for patients who are either untreated or treated too late (Bahk and Marth 1990). The mechanisms by which *L. monocytogenes* causes listeriosis are poorly understood (Marth 1988).

No seasonal trend in the incidence of listeriosis was found in a 1986 study in the US that closely monitored cases of listeriosis in a population of 34 million people (Gellin et al. 1991). Whether or not the incidence of *L. monocytogenes* on poultry meat exhibits seasonal trends has not yet been investigated.

The infectious dose for listeriosis is unknown (Breer and Schopfer 1988). Reported incubation times vary from a few days to 2-3 months (Bille and Doyle 1991). People can be infected with *L. monocytogenes* and not have any clinical symptoms. Most US. consumers are exposed to *Listeria* organisms at least once a week, but the exposures must be moderate or else many thousands more cases of listeriosis would occur annually (Anonymous 1993). Workers in the UK obtained second and third specimens from 11 nonsymptomatic people who were excreting this organism, and it was found that the average time between isolation and failure to isolate *L. monocytogenes* was about 2.5 weeks (Kwantes and Isaac 1975). Between 2% and 20% of animals and humans are transient carriers of the organism (Bille and Doyle 1991). Stool-carriage studies have reported that asymtomatic intestinal carriage of *L. monocytogenes* occurs in 1% to 5% of humans (Baron et al. 1994).

There is scant information concerning the incidence of listeriosis, but estimates indicate that there are about 1,600 cases annually in the United States (Lovett 1989).

A recently published analysis of surveillance data collected in 1986 estimated that 1,700 cases of listeriosis and 450 deaths due to listeriosis occurred in the United States in 1986 (Gellin et al. 1991). The incidence of sporadic cases of listeriosis in countries that report the illness is typically about 5-8 cases per million people (Bille and Doyle 1991). In foodborne outbreaks the incidence rate may rise to 50 per million people (Bille and Doyle 1991).

Evidence that the foodborne route is the primary mode of transmission of *L. monocytogenes* from its environmental reservoir to humans is continuing to mount (Schlech 1988). Outbreaks of listeriosis have been traced to coleslaw (cabbage), poultry, turkey frankfurters, mushrooms, soft cheeses, chocolate milk, milk, ice cream, and similar dairy products. *L. monocytogenes* can be shed in milk from mastitic cows. A Nova Scotia outbreak was traced to coleslaw made from cabbages that were grown in fields fertilized with sheep manure harboring *L. monocytogenes* (Bahk and Marth 1990).

Transmission of *L. monocytogenes* from animals to humans has been documented after people have worked with diseased animals (Marth 1988). Up until 1987, no outbreaks of human listeriosis had been directly attributed to processed poultry (Bailey and Fletcher 1987). However, in 1988 a woman contracted listeriosis from a cooked and chilled chicken purchased in a supermarket, which caused her to deliver a stillborn 23-week fetus (Kerr et al. 1988). Phage types of the cooked chicken and the post mortem liver were both 144/3274/2671/108/340. Another case of listeriosis was traced to turkey frankfurters (Johnson et al. 1990).

2.2. Description of *L. innocua* and *L. monocytogenes*

Listeria are small, short, regular, facultatively anaerobic gram-positive rods with flagella that give them motility when grown at 20-25 °C. Currently there are seven species of *Listeria*, which include *L. monocytogenes, L. innocua, L. welshimeri, L. ivanovii, L. seeligeri, L grayi* and *L. murrayi* (Jones and Seeliger 1992). Coccoid forms of *Listeria* are 0.4-0.6 μm in length and 0.4-0.5 μm in diameter (Seeliger and Jones 1986). The list of biochemical properties in Table 1 demonstrates that *L. innocua* and *L. monocytogenes* have many similar biochemical characteristics.

2.2.1. PATHOGENICITY

L. innocua, L. welshimeri, and *L. seeligeri* are not considered to be virulent (Bahk and Marth 1990). *L. ivanovii* has rarely been reported as being involved in

human pathology (Lovett and Twedt 1988), but it is pathogenic for animals (Prentice and Neaves 1992). There have been a few reports suggesting that other species have caused human illness, but only *L. monocytogenes* is currently believed to be pathogenic for humans (Bahk and Marth 1990).

Not all strains of *L. monocytogenes* are pathogenic. Nonhemolytic avirulent strains of *L. monocytogenes* have been identified that respond identically to all identification tests with the exception of the hemolysin reactions and virulence tests (Pine et al. 1987). Some environmental strains of *L. monocytogenes* have appeared to be avirulent when injected into mice (Schlech 1988). Pathogenic evaluation of 22 *Listeria* isolates from poultry showed that 17 were pathogenic, and the other 5 were less virulent (i.e. only killed some of the mice in the test group) (Bailey et al. 1989). Of 27 milk samples that tested positive for *L. monocytogenes*, 25 harbored pathogenic strains (Bahk and Marth 1990).

2.2.2. RESERVOIRS AND SURVIVAL IN THE ENVIRONMENT

Listeria are widely distributed in nature and have been isolated from soil, vegetation, silage, sewage, slaughterhouse waste, humans, 43 species of domestic and wild animals, 22 species of birds, water, animal feed, fresh and frozen poultry, seafood (oysters, squid and crab), pond trout, ticks, flies and plants such as vegetables, corn, cereals, soybeans and weeds (Gregorio and Eveland 1975; Jones and Seeliger 1992; Marth 1988; Seeliger and Jones 1986; Wong et al. 1990). All seven *Listeria* species have been isolated from fresh poultry products (Johnson et al. 1990). Broilers can be fecal carriers of *Listeria* which can infect the litter, the environment, and even man and other animals (Bailey et al. 1989).

L. monocytogenes can survive more than 3 months in fodder, 6 months in dry straw, 2 years in dry feces, 12 days in top soil exposed directly to sunlight, 2 years in damp soil, and 44 days on glass beads in sealed tubes at room temperature (Lovett 1989). In some instances, *L. monocytogenes* has been observed to survive in cheddar cheese for more than 400 days (Doyle 1988).

Table 1 - Biochemical Characteristics of *L. innocua* and *L. monocytogenes*

Characteristics	*L. monocytogenes*	*L. innocua*
Irregular rods with uneven staining	-	-
Formation of capsules	-	-
β-Hemolysis [a]	+	-
Hemolysis on horse blood	+	-
Hemolysis of calf erythrocytes	+	-
CAMP-test (*Staphylococcus aureus*)	+ [b]	-
CAMP-test (*Rhodococcus equi*)	-	-
Acid production from:		
Amygdalin	+	+
L-Arabinose	-	-
Cellobiose	+	+
Dextrin	d [c]	-
Dextrose	+	+
Esculin	+	+
Fructose	+	+
Galactose	d	-
Glucose	+	+
Glycerol	+	+
Glycogen	-	-
Lactose	d	+
D-Lyxose	-	-
Maltose	+	+
Mannitol	-	-
Mannose	+	+
Melezitose	d	d
Melibiose	-	-
α-Methyl-D-glucoside	+	+
α-Methyl-D-mannoside	+	+
Methyl-manno-pyranoside	+	+
L-Rhamnose	+	d
Salicin	+	+
Sorbitol	d	-
Soluble starch	-	-
Sucrose	-	d
D-Xylose	-	-
Utilization of citrate	-	-
Indole	-	-
Triple Sugar Iron Agar (TSIA)	A/A [d]	A/A
H_2S Production	-	-
Voges-Proskauer	+	+
Methyl Red	+	+

Table 1 - Continued

Characteristics	*L. monocytogenes*	*L. innocua*
Hydrolysis of:		
Casein	-	-
Cellulose	-	-
Esculin	+	+
Gelatin	-	-
Hippurate	+	+
Milk	-	-
Starch	d	d
Tryosine	-	-
Urea	-	-
Xanthine	-	-
Enzyme Production:		
Arginine decarboxylase	-	-
Arginine dihydrolase	-	-
Catalase	+	+
β-D-galactosidase	+	+
Lecithinase	d	d
Lysine decarboxylase	-	-
Phenylalanine deaminase	-	-
Phosphatase	+	+
Ornithine decarboxylase	-	-
Oxidase	-	-
Sulfatase	-	-
Reduction NO_3 to NO_2	-	-
Tumbling Motility	+	+
Umbrella Motility	+	+
Pathogenicity for mice	+	-
Mol % G + C	37-39 (T_m) 38 (Bd)	36-38 (T_m) 38 (Bd)
Major peptidoglycan diamino acid	*meso*-DAP	*meso*-DAP
Major menaquinone	MK-7	MK-7
Acid-fast	-	-
Fatty Acid Composition	S,A,I [e]	S,A,I

[a] Not all strains of *L. monocytogenes* exhibit β-hemolysis--the type strain ATCC 15313 is nonhemolytic on horse, sheep and bovine blood.

[b] Of 30 strains listed, ATCC 15313, the type strain, did not give a positive reaction.

[c] d, 11-89% of the strains are positive; +, over 90% of the strains are positive; -, over 90% of the strains are negative

[d] A/A - Acid slant over acid butt - lactose fermenter

[e] S, straight-chain saturated; A, *ante iso*-methyl-branched; I, *iso*-methyl branched

Sources: (Bille and Doyle 1991; Lovett 1988; McLauchlin 1987; Seeliger and Jones 1986; Skovgaard and Morgen 1988)

2.2.3. TEMPERATURE, pH AND GROWTH CONSIDERATIONS

The optimum temperature for the growth of *Listeria* species ranges from 30 to 37 °C with temperature limits ranging from 1-45 °C (Seeliger and Jones 1986). *Listeria* grow between pH 6 and 9, and are capable of growing in nutrient broth supplemented with up to 10% (w/v) NaCl (Seeliger and Jones 1986). It does not appear that detailed studies of temperature and pH ranges for growth of *L. innocua* have been undertaken. This is likely due to the fact that *L. innocua* is a nonpathogenic species. A wealth of information about pH and temperature ranges for growth of *L. monocytogenes* is available.

Optimal growth of *L. monocytogenes* occurs between 30 and 37 °C (Petran and Zottola 1989; Wilkins et al. 1972). This organism is one of the few foodborne pathogens that can grow at refrigeration temperatures (Doyle 1988). Until recently, it was thought that the organism would only grow in temperatures ranging from 2.5 to 42 °C (Brackett 1988). However, a more recent publication reported that the minimum temperatures at which three strains of *L. monocytogenes* grew ranged from -0.1 to -0.4 °C (Walker et al. 1990) and the authors note that growth at lower temperatures cannot be ruled out. Temperatures near 0 °C gave significantly longer lag phases on growth curves (Walker et al. 1990). Significant growth rates for *L. monocytogenes* were found to occur on artificially inoculated processed chicken slices that were refrigerated at 4.4 °C. An increase of 10^3 to 10^5 CFU/g occurred within 4 weeks (Glass and Doyle 1989). Carbon dioxide atmospheres have been shown to inhibit the growth of *L. monocytogenes* at a refrigeration temperature of 6 °C (Hart et al. 1991).

At the other end of the spectrum, experiments have been conducted to determine what level of heat inactivates *L. monocytogenes*. Chicken breast surfaces were inoculated with approximately 10^5-10^6 microorganisms/g of chicken and cooked until internal temperatures reached a range from 65.6 to 82.2 °C using dry heat (Carpenter and Harrison 1989) and moist heat (Harrison and Carpenter 1989). Survivors were encountered at each cooking temperature, but cooking the chicken to an internal temperature of 73.9 °C gave a 4 to 5 log reduction of the organism. Since raw chicken would not be expected to harbor populations of *L. monocytogenes* the size of the initial inoculum, cooking to an internal temperature of 73 °C should be adequate (Carpenter and Harrison 1989). This finding is supported by others who have studied the heat resistance of *L. monocytogenes* and state that cooking food to an internal temperature of 70 °C for 2 minutes is adequate to ensure destruction of

L. monocytogenes (Mackey and Bratchell 1989). It should be noted that meats that are heated up slowly may contain *L. monocytogenes* that have increased resistance to heat due to heat shock response (Bunning et al. 1990; Farber and Brown 1990).

All *Listeria* strains grow best at neutral to slightly alkaline pH with lower and upper pH limits for some strains ranging from 5.5-9.6 (Seeliger and Jones 1986). The ability of 16 strains of *L. monocytogenes* to grow in a nutrient medium acidified with HCl to selected pH values showed that for growth at 4 °C the minimum pH was 5.23, while at 30 °C the minimum pH was 4.39 (George et al. 1988). At low pH (4.5), faster growth rates occurred under anaerobic conditions at temperatures of 19 °C and 28 °C than under aerobic conditions (Buchanan and Klawitter 1990).
L. monocytogenes was able to survive at 37 °C with anaerobic conditions, but did not survive in an aerobic environment at 37 °C (Buchanan and Klawitter 1990). Optimum growth occurs at pH 7 (Petran and Zottola 1989).

The best incubation temperature for sublethally injured *Listeria* that had been exposed to heat below inactivation temperatures was found to be 25 °C (Lovett 1988). This was true of plating cultures and was also thought to be true of enrichment cultures.

2.2.4. GROWTH MEDIA

Once *Listeria* species have been isolated, they grow well on usual nutrient media (Seeliger and Jones 1986). The organism may be preserved for some months in screw-capped containers by stab inoculation into a nutrient agar such as Tryptose Agar (Difco) or other similar media (Seeliger and Jones 1986).

Several different media have been used to grow *Listeria* for use in experiments. The medium used to determine the ability of *L. monocytogenes* strains to grow at low pH contained Trypticase soy broth, glucose and yeast extract (George et al. 1988). *L. monocytogenes* strains used in virulence experiments were grown in Trypticase soy broth with 0.6% yeast extract (TSBYE) (Datta and Wentz 1989). Strains used for phage typing were inoculated into tryptose-phosphate broth and incubated at 30 °C until the log-phase of growth was reached (generally 3 to 5 hours) (Ortel 1989). Six *Listeria* strains used in disinfection experiments were grown in tryptic soy broth and on tryptic soy agar (Van de Weyer et al. 1993).

2.2.5. IDENTIFICATION

In addition to the biochemical tests listed in Table 1, serotype analysis can be helpful in identification of isolates. Currently *Listeria* strains have been organized into

16 serovars (1/2a, 1/2b,1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 5, 6a, 6b, 7) (Seeliger and Langer 1989). *L. innocua* strains have the antigenic composition of serogroups 3, 4ab, 6a, and 6b (Jones and Seeliger 1992; Seeliger and Jones 1986).
L. monocytogenes strains have been organized into twelve serotypes (Lovett 1989). Virulent strains of the organism are found in serotypes 1, 3, 4 and 7, but serotype analysis is not sufficient to determine pathogenicity because some environmental strains of *L. monocytogenes* appear to be avirulent when injected into mice (Schlech 1988).

Pathogenic serotypes from human sources in Britain included 1/2a, 1/2b, 1/2c, 3, 4b and other serotype 4 strains (McLauchlin 1987). Serotypes 1/2a, 1/2b, 1/2c, 3a, 3b and 4b were found in 162 clinical isolates from humans in a US study (Gellin et al. 1991).

Phage typing has been proposed as another means of identifying *Listeria* isolates and 27 phages were used to type 645 of 823 strains (78%) (Audurier et al. 1984). More recently 1508 (61.1%) of 2470 strains were typed (Audurier and Martin 1989) A greater percentage of serogroup 4 were typable than serogroup 1. No information exists concerning the comparative virulence of different phage types of the same serotype (Schlech 1988).

In general, the tests utilized in biotyping *Listeria* species have demonstrated greater ability to distinguish between virulent and avirulent strains than serotyping and phage typing. These tests can also distinguish between virulent and avirulent strains of *L. monocytogenes* (Schlech 1988).

2.2.6. SAMPLING AND ISOLATION CONCERNS

Since the concentration of *L. monocytogenes* is usually fairly low in foods, most isolation methods use an enrichment stage before isolation (Lovett 1988). Cold enrichment is one of the oldest and simplest isolation methods. A sample is added to tryptose broth, followed by incubation at 4 °C. Storage at this temperature retards growth of many organisms but allows growth of *L. monocytogenes* (Bahk and Marth 1990). The culture is sampled at intervals for 4-8 weeks, plated and incubated 48 hours at 35 °C. Biochemical tests and typing of the isolates may be done.

Since direct plating procedures do not reliably isolate *Listeria* spp., they are typically used in conjunction with a prior enrichment. Recently developed methods that use combinations of nonselective preenrichments and/or selective enrichments followed by plating to non-selective and /or selective agars have been reviewed (Slade

1992). Comparative studies have shown that no single isolation method is effective in detecting all *Listeria*-positive raw milk, raw meat, vegetable and food samples (Slade 1992). For optimal detection of *Listeria* it may be necessary to utilize several isolation protocols.

Selective agars have been developed that have improved the isolation of *Listeria*. Two such agars are PALCAM (van Netten et al. 1989) and Oxford Agar (OA). Of the two, Oxford agar is more widely used (Jones and Seeliger 1992). PALCAM medium is preferred in Europe, while LiCl phenyl ethanol moxalactam (LPM) agar and Oxford agar are the most widely used in North America (Slade 1992).

A recently developed method to detect *L. monocytogenes* makes use of DNA amplification (Deneer and Boychuk 1991). The method does not require prior enrichment. However, this method is only semiquantitative and further refinement is necessary before quantitative results can be obtained.

Enzyme-linked immunoassay (ELISA) is another detection method. In a comparison of the ELISA method to the culture method, 29 of 102 cooked chicken samples were positive for *Listeria* by the culture method (Kerr et al. 1990). Of the 29 samples positive by culture, only 24 were positive by ELISA. Two false negative samples were subcultured and incubated for a longer period of time after which the ELISA test was positive. The authors suggested that where low numbers of *Listeria* spp. are present, longer incubation times will help reduce false negatives by the ELISA method.

2.3. *L. innocua* as a Surrogate for *L. monocytogenes*

Laboratory procedures are much more involved and time consuming when dealing with pathogenic bacteria compared with nonpathogenic bacteria. For the purposes of preliminary experiments, it is desirable to be able to work with a nonpathogenic *Listeria* species (*L. innocua*) that is closely enough related to the pathogenic species of interest (*L monocytogenes*) so that the results can be tentatively extended to apply to the pathogenic species. The following discussion shows that *L. innocua* is genetically and phenotypically closely related to *L. monocytogenes* and also that *L. innocua* responds to many disinfectants in a similar fashion to *L. monocytogenes*.

2.3.1. GENOTYPIC AND PHENOTYPIC RELATEDNESS

The genotype of an organism refers to its genetic makeup, to its collection of genes, its entire DNA (Tortora et al. 1989). Each strand of DNA making up the double helix consists of alternating sugar and phosphate groups called nucleotides. The rungs of the ladder are made up of nucleotide bases called adenine (A), thymine (T), cytosine (C), and guanine (G). Base A is always paired with T and G is always paired with C (Tortora et al. 1989).

The mole percent guanine plus cytosine content (mol% G + C) is useful in distinguishing between strains of bacteria where mol% G + C values range from 25-75 (Johnson 1986). From Table 1 the mol% G + C values obtained from the Buoyant Density Method were 38 for both *L. monocytogenes* and *L. innocua*. The mol% G + C from the Thermal Denaturation Method ranged from 37 to 39 for *L. monocytogenes*, and from 36 to 38 for *L. innocua*. These mol% G + C values indicate that there is a high degree of genotype relatedness between the two species.

However, just because two organisms have similar mol% G + C values does not mean that they are necessarily closely related because mol% G + C values do not take into account the linear arrangement of the nucleotides in the DNA (Johnson 1986). The genotype represents the potential properties of an organism but not the properties themselves. So examination of phenotype relatedness is necessary.

Phenotype refers to the actual, expressed properties of an organism such as its ability to perform a particular chemical reaction (e.g. hydrolysis of sucrose). The biochemical characteristics listed in Table 1 show that there is a very high degree of phenotypic relatedness between *L. innocua* and *L. monocytogenes*. The results from 59 of 70 biochemical tests in Table 1 were similar for the majority of *L. innocua* and *L. monocytogenes* strains tested. In only 5 tests based on hemolysis and pathogenicity was there a sharp difference between the two species. Most *L. monocytogenes* strains are pathogenic and are positive for most hemolysis tests, while *L. innocua* are negative for both. In six of the tests minor differences were reported for the two species. For example, 11-89% of the *L. monocytogenes* strains produce acid from dextrin, while only 0-10% of the *L. innocua* strains do so.

Because the biochemical characteristics of *Listeria* species are so similar, *Bergey's Manual of Systematic Bacteriology* advises that "due to the high phenotypic similarity between strains of *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri* and *L. welshimeri*, it is particularly important that new isolates are examined for hemolytic activity and for experimental pathogenicity in mice" (Seeliger and Jones

1986). In addition it should be noted that, since some *L. monocytogenes* strains are nonhemolytic, they can only be differentiated from *L. innocua* by serotyping, phage typing, or DNA hybridization (Pine et al. 1987).

2.3.2. COMPARISON OF RESPONSE TO DISINFECTANTS

Several research projects have compared the response of *L. innocua* and *L. monocytogenes* strains to a range of disinfectants. Although there are minor differences between the two species for some disinfectants, overall their susceptibility to disinfectants is remarkably similar.

A 1989 study examined the effect of 11 disinfectants (at several concentrations) on 3 strains of *L. monocytogenes* and one strain of *L. innocua* in four media (water, water and whey, water and milk, and water and salt) (Cordier et al. 1989). A total of 23 experiments were carried out in which each of the 4 strains were exposed to the same concentration of disinfectant in each of the four media giving 92 individual results. Samples were collected at 1, 5 and 15 minute intervals and analyzed by the impedimetric method to indicate whether the bacteria were inactivated in less than 1 minute, or were destroyed after 1, 5 or 15 minute contact times. The disinfectants consisted of compounds that included one the following active agents: peroxide, acids, chlorine, iodine, formaldehyde, quaternary ammonium, aldehydes, alkylamido-alkylglycine, chlorhexidine digluconate, and a cationic compound.

In 58 of the 92 individual results, the contact time for the inactivation of the *L. innocua* strain was the same as 1 or more of the *L. monocytogenes* strains and greater than the remainder. In 11 of 92 outcomes, the *L. innocua* strain was more difficult to inactivate than the other 3 *L. monocytogenes* strains, while in 22 of 92 results, the *L. innocua* strain survived for a shorter period of time than some or all of the *L. monocytogenes* strains. Of these 22 results, in 11 cases the *L. innocua* strain was only inactivated more easily than 1 of the *L. monocytogenes* strains; in 6 instances *L. innocua* was more easily inactivated than 2 *L. monocytogenes* strains; and on 5 occasions *L. innocua* was more easily inactivated than all 3 of the *L. monocytogenes* strains.

The results from the experiments using water as the suspension media are the most relevant for the work done in this thesis. Eleven of the 23 trials carried out in water gave identical results for all 4 *Listeria* strains. In 5 of the trials *L. innocua* survived as long as the most resistant *L. monocytogenes* strain and longer than the less

resistant *L. monocytogenes* strains. In 7 of the trials *L. innocua* was more easily inactivated than 1, 2 or 3 of the *L. monocytogenes* strains.

Other workers examined the efficacy of 12 disinfectants {ethanol, sodium hypochlorite (60 μg/mL and 10 μg/mL), sodium dichloroisocyanurate (60 μg/mL and 10 μg/mL), chloramine-T, sodium hypochlorite with 4% methylethanol, phosphoric acid, povidone-iodine, iodophor, glutaraldehyde, glutaraldehyde-phenate, formaldehyde, quaternary ammonium compound (10% and 3.88%)} against one *L. innocua* strain and two strains of *L. monocytogenes* in suspensions of tryptic soy broth (TSB) and whole pooled human serum (Best et al. 1990). Of the 23 trials reported, the $\log_{10}$ reduction results for 14 trials was similar for the two species. In two trials, the *L. innocua* strain was slightly more resistant, but the difference between the $\log_{10}$ reduction of the two species was less than 1 $\log_{10}$ cycle. In 6 of the trials the *L. monocytogenes* strains appeared to be slightly more difficult to inactivate with less than 1 log separating the $\log_{10}$ reduction between species in 4 of the 6 trials and less than 2 logs separating the results in the other two trials. These results indicate that for the majority of the tests the two *Listeria* species responded in a similar fashion to a large number of disinfectants.

In a more recent study, 1 *L. innocua*, 1 *L. welshimeri*, and 4 *L. monocytogenes* strains were exposed to 9 disinfectants for a contact time of 5 minutes (Van de Weyer et al. 1993). The disinfectants were commercially available products intended for use in kitchens or the dairy industry. In some cases, the researchers could not obtain the exact formula of the disinfectant. The active agents (with test concentrations in parentheses) in the disinfectants included the following: quaternary ammonium compound (0.025% and 0.036%), disochlorine (0.3% active chlorine), substituted phenols (0.5%), ethanol and substituted phenol (not diluted), alcohols (not diluted), aldehydes (0.5%), cationinc surface-active agents plus glyoxal (0.5%) and anionic and non-ionic surface-active agents plus aldehyde and complexing agents (0.5%). The six *Listeria* strains reacted in the same way to the different classes of disinfectants and were not particularly resistant to the disinfectants under these experimental conditions. In tests where organic matter was not present, there was a 6.2 to 7.2 log inactivation of *Listeria* species ($N_0 = 5 \times 10^8$ to 5×10^9 organisms/mL). The tests were also applied to *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Both of these bacteria responded similarly to *Listeria* to 5 of the disinfectants and were more resistant than the *Listeria* strains to 4 of the disinfectants.

The results from the above studies indicate there is not a significant difference in the ability of the *L. innocua* strains and the *L. monocytogenes* strains to survive exposure to a range of disinfectants at different concentrations. This lends support to the idea that *L. innocua* may be a suitable surrogate for *L. monocytogenes* in disinfection experiments.

2.4. Incidence of *L. innocua* and *L. monocytogenes* in the Poultry Industry

Although this review focuses on the incidence of *L. innocua* and *L. monocytogenes* on poultry products, it should be noted that *Listeria* spp. have been isolated from a large number of other meat products. These include raw meat (beef, lamb and pork); minced meat (beef, lamb, and pork); cattle hides; lamb pelts; bacon; air dried meat; uncooked ham; salami; sausage (beef, pork and smoked); pate (meat, fish and chicken); and raw fish (Breer and Breer 1988; Lowry and Tiong 1988; MacGowan et al. 1994). *Listeria* spp. have also been isolated from knives and work surfaces in beef and lamb cutting plants (Lowry and Tiong 1988).

2.4.1. INCIDENCE OF *L. MONOCYTOGENES* IN POULTRY PRODUCTION

The incidence of *L. innocua* on poultry farms has not been investigated, and information about *L. monocytogenes* during poultry production is scarce. Some poultry houses remain free of detectable *L. monocytogenes* (Pardon et al. 1994). So in spite of the organism's wide distribution, contamination by *L. monocytogenes* should not be considered as unavoidable in live poultry production (Pardon et al. 1994).

In Sweden, *L. monocytogenes* was not found on processed chicken in one plant where samples came from 6 breeders (Loncarevic et al. 1994). However in another Swedish plant the organism was isolated from over 50% of samples that came from 23 breeders. Unfortunately, the investigators grouped their findings by sampling day and did not indicate if flocks from some of the 23 breeders were free of the organism.

In Norway, processed chicken samples were collected from 12 flocks in two plants (Rorvik and Yndestad 1991). In one plant, samples from processed chicken meat representing 9 flocks were positive for *L. monocytogenes*, while in another plant processed chicken meat from 3 flocks were negative.

2.4.2. INCIDENCE OF *L. INNOCUA* AND *L. MONOCYTOGENES* IN POULTRY PLANTS

Recently several studies have determined the incidence of *Listeria* in the poultry processing plant environment. The results from a Danish report (Skovgaard and Morgen 1988) are of limited usefulness. Only single samples were taken from the scald tank and giblets cooling water and only two samples were taken from the spin-chiller. All of them tested negative for *L. monocytogenes*. The authors chose to pool neck-skin samples from 10 chickens into a composite sample. In this manner 8 composite samples representing 80 chickens were collected from air-cooled carcasses, while 9 composite samples were collected from spin-chilled birds. *L. monocytogenes* was found in 3 of 8 air-cooled samples (37.5%), while 3 of 9 (33.3%) spin-chilled samples tested positive. This method of analysis gives little information about the true incidence of *L. monocytogenes* since a small number of contaminated birds could account for the incidence level detected.

A study with a better design was conducted in the UK at a chicken processing plant (Hudson and Mead 1989). Samples were collected on three separate visits to the plant and the results are presented in Table 2. *L. monocytogenes* was isolated in all three visits from the automatic carcass opener, but not as frequently from the neck-skin trimmer and conveyor to the packaging area. It appeared that birds were not contaminated immediately after slaughter up to the evisceration station. The authors concluded that the occurrence of *L. monocytogenes* on the finished carcasses (50% of those examined) was attributable to contamination of processing equipment and consequent cross-contamination (Hudson and Mead 1989) while acknowledging that the isolation method used could have been inadequate at the first two sampling sites for samples heavily contaminated with other organisms.

The incidence of *Listeria* in a California chicken processing plant was reported (Genigeorgis et al. 1989). On each of four trips to a chicken processing plant, a single sample was collected from scalding tank water overflow (SWO), feather picker drip water (FPDW), recycled water for cleaning gutters (RWCG), chiller incoming water (CIW), chiller overflow water (CWO), caeca (CC) and the last part of the large intestine (LI) from the evisceration line, neck skins (NK), livers (LV), hearts (HT), and mechanically deboned meat (MDM). The samples were analyzed for *Listeria* species. The results for *L. monocytogenes* and *L. innocua* are given in Table 3. Most of the sampling sites tested negative for *L. monocytogenes*. The organism was isolated from 12.5% of the chiller water overflow samples, and 6.3% of the recycled water samples,

indicating the potential for cross-contamination. The organism was not isolated from livers, hearts and neck skin samples at the plant, but at the supermarket level *Listeria* spp. were isolated from 53% of neck skin samples, and from 25% of the heart samples (Genigeorgis et al. 1989). In order to examine this discrepancy, liver, wing and leg samples were collected on six trips to the plant immediately after chilling, and from the packaged final product. The post-chill samples were negative for *L. monocytogenes* while from the packaged products the organism was isolated from 33.3% of the liver samples, 70% of the wing samples, and 37% of the leg samples. *L. monocytogenes* was also isolated from 35% of the samples collected from the hands and gloves of plant workers at the leg and wing cutting station and the leg and wing packaging station. This data demonstrated beyond doubt the potential contribution of handlers to cross-contamination (Genigeorgis et al. 1989).

Table 2 - Occurrence of *L. monocytogenes* at Different Stages of a UK Processing Plant

Type of Sample	Type of Sample	No. of Samp./Visit	Visit 1	Visit 2	Visit 3
Transport crates	Swab	3	-	-	-
Neck skin of freshly killed birds	Neck skin	10	0/10	0/10	0/10
Scald water	1 liter	1	-	-	-
Defeathering machines	Swab	3	-	-	-
Feathers from defeathering machine	Feathers	1	-	-	-
Automatic carcass opener	Swab	1	+	+	+
Caeca during evisceration	Skin	10	0/10	0/10	0/10
Evisceration-line drain	1 liter	1	+	+	-
Neck-skin trimmer	Swab	1	-	+	+
Conveyor to chiller	Swab	1	-	-	-
Chiller water	1 liter	1	-	-	-
Conveyor to packing area	Swab	1	-	+	-
Neck skin prior to packaging	Skin	10	1/10	8/10	6/10

+ = Positive for *L. monocytogenes*; - = negative

Source: (Hudson and Mead 1989)

Table 3 - Prevalence of *Listeria* at Various Stages in a Chicken Processing Plant

L. monocytogenes

	Ftr	SWO	FPDW	CIW	CWO	RWCG	NK	LV	HT	CC	LI	MDM	Total
# Pos/# Samp[a]	0/16	0/16	0/16	0/16	2/16	1/16	0/12	0/16	0/16	0/16	0/16	3/16	6/188
% Pos.	0	0	0	0	12.5	6.3	0	0	0	0	0	19	3.2

L. innocua

	Ftr	SWO	FPDW	CIW	CWO	RWCG	NK	LV	HT	CC	LI	MDM	Total
# Pos/# Samp[a]	0/16	0/16	3/16	0/16	0/16	5/16	0/12	0/16	4/16	0/16	0/16	2/16	14/188
% Pos.	0	0	18.8	0	12.5	31.3	0	0	25	0	0	12.5	7.4

[a] = Number positive / total sample. ND = Not done, Ftr = Feathers, SWO = scalding water overflow, FPDW = feather-picker drip water, CWO = chiller water overflow, RWCG = recycled water for cleaning gutters, NK = neck skin, LV = livers, HT = hearts, CC = cacca, LI = last part of the large intestine, MDM = mechanically deboned meat

Source: (Genigeorgis et al. 1989)

A similar type of investigation was carried out for a California turkey processing plant (Genigeorgis et al. 1990). Samples were collected from similar sites in the plant on three visits. The results are presented in Table 4. *L. monocytogenes* was found in the feather picker drip water (6.7% of samples) and recycled water for cleaning gutters (13.3% of samples), indicating the potential for cross-contamination. *L. innocua* was not isolated in the plant. *L. welshimeri* was isolated from 7 of 225 samples.

Table 4 - Prevalence of *L. monocytogenes* at Various Stages in a Turkey Processing Plant

Trip	Ftr	SWO	FPDW	CWO	RWCG	NK	LV	HT	CC & LI	MDM	Total
1	0/10[a]	0/5	1/5	0/5	0/5	0/10	0/10	0/10	0/10	1/5	2/75
2	0/10	0/5	0/5	0/5	2/5	0/10	0/10	0/10	0/10	0/5	2/75
3	0/10	0/5	0/5	0/5	0/5	0/10	0/10	0/10	0/10	1/5	1/75
Total	0/30	0/15	1/15	0/15	2/15	0/30	0/30	0/30	0/30	2/15	5/225
% Pos.	0	0	6.7	0	13.3	0	0	0	0	13.3	2.2

[a] = Number positive / total sample. Ftr = Feathers, SWO = scalding water overflow, FPDW = feather-picker drip water, CWO = chiller water overflow, RWCG = recycled water for cleaning gutters, NK = neck skin, LV = livers, HT = hearts, CC = cacca, LI = last part of the large intestine, MDM = mechanically deboned meat

Source: (Genigeorgis et al. 1990)

Another component of this investigation examined 180 retail samples of fresh turkey parts from two companies (A and B) for the presence of *Listeria* (Genigeorgis et al. 1990). *L. monocytogenes* and *L. welshimeri* were isolated from 15% of the samples, while *L. innocua* was found on 1.7% of the samples. While 23% of retail wing, drumstick and tail samples from Company A harbored *Listeria* spp., the neck skin, liver and heart samples in the plant tested negative. To find factors contributing to this discrepancy, a further analysis was carried out. On three trips to plant A, 30 drumstick, 30 wing and 30 tail samples were collected at two sites: 1) immediately after chilling, and 2) from the final packaged product. *L. monocytogenes* was found in only one wing sample after chilling, while 13.3% of the packaged wings and 6.7% of the packaged legs tested positive. The packaged tails tested negative. Samples were also collected from the hands and gloves of workers at three post-chill stations, and 11 of 90 (12.2%) tested positive. Again this demonstrates the potential for handlers to cross-contaminate products with *L. monocytogenes*.

A recent Swedish study examined pre-chill and post-chill broiler carcasses for the presence of *L. monocytogenes* and *L. innocua* (Loncarevic et al. 1994). Samples consisted of neck skins from 5 broilers that were pooled. From each pooled sample 25 g were cut out and macerated with 225 mL of *Listeria* enrichment broth in a stomacher. The results are presented in Table 5.

Table 5 - Prevalence of *Listeria* spp. in Pooled Samples of Broilers Neck Skins Collected Before and After Chilling

Plant	Number of Pre-chill Samples	Number of Post-chill Samples	Number (%) of Samples Positive			
			L. monocytogenes		*L. innocua*	
			Pre-chill	Post-Chill	Pre-chill	Post-Chill
A	62	62	n.d.*	36 (58)	12 (19)	2 (3)
B	99	100	n.d.	n.d.	39 (39)	6 (6)

* n.d. = Not detected

Source: (Loncarevic et al. 1994)

In Plant A, *L. monocytogenes* was not isolated from 62 pre-chill pooled samples, but 36 (58%) of the post-chill samples tested positive. This indicated that a small number of contaminated birds could cause cross-contamination to occur in the chiller tank.

2.4.3. INCIDENCE OF *LISTERIA* ON POULTRY PRODUCTS

A few of the studies that have primarily investigated the incidence of *L. monocytogenes* on poultry meat products have also reported the incidence of *L. innocua*. The findings are listed in Table 6.

Table 6 - Incidence of *L. innocua* in Poultry Products

Origin	Type of Poultry Product	Type of Sample Taken	No. of Samp.	No. Pos.	% Pos.	Reference
Switzer-land	Fresh chicken	Not specified	56	20	32.1	(Breer and Schopfer 1988)
New Zealand	Retail poultry portions	25-100g samples	25	12	48.0	(Lowry and Tiong 1988)
Denmark	Neck skin	2-3 g of neck skin - Stomacher	17	16	94.1	(Skovgaard and Morgen 1988)
Canada	Retail chicken legs	25 g of sample into enrichment broth	16	8	50.0	(Farber et al. 1989)
U.S.A.	Fresh retail chicken parts	Skin portion removed and rinsed	160	42	26.3	(Genigeorgis et al. 1989)
U.S.A.	Fresh retail turkey parts	Skin removed and rinsed	180	3	1.7	(Genigeorgis et al. 1990)
U.K.	Precooked/chilled retail chicken	25 g sample - homogenized	102	1	0.98	(Kerr et al. 1990)
Austra-lia	Frozen retail chicken	Rinse samples	80	14	17.5	(Varabioff 1990)
U.K.	Retail raw chicken products	25 g sample - homogenized	32	12	37.5	(MacGowan et al. 1994)

The incidence of *L. monocytogenes* on poultry products has been investigated in several countries. The findings are listed in Table 7. In evaluating the significance of the findings in Table 7 for potential risks posed to humans, it is helpful to look at the serotypes reported for the strains isolated. Serotypes of *L. monocytogenes* isolated from poultry in a US study included 1/2b, 1/2c, and 3b (Bailey et al. 1989). In British studies, serotypes 1, 1/2, 3a, 3b, 3c, 4b and 4d have been found (Gitter 1976; Kwantes and Isaac 1975; Pini and Gilbert 1988), while in New Zealand serotypes 1/2a,

1/2b 1/2c, and 4b were isolated from raw poultry, beef and pork samples (Breer and Breer 1988). Serotypes 1 and 4 have been isolated from processed poultry in the U.K. (Kerr et al. 1990), Australia (Varabioff 1990), Norway (Rorvik and Yndestad 1991) and Taiwan (Wong et al. 1990). From this limited information, it appears that most of the poultry serotypes match the human serotypes.

Several of the investigators listed in Table 7 either did not carry out serotyping of the strains they isolated or did not report them (Bailey and Fletcher 1987; Genigeorgis et al. 1989; Genigeorgis et al. 1990; Hudson and Mead 1989; Kwantes and Isaac 1971; Loncarevic et al. 1994). None of the authors reported phage types.

Table 7 - Incidence of *L. monocytogenes* on Poultry Products

Origin	Type of Poultry Product	Type of Sample Taken	No. of Samp.	No. Pos.	% Pos.	Reference
U.K.	Fresh chicken	Swab	35	20	57	(Kwantes and Isaac 1971)
U.K.	Fresh chicken	Swab - inside and outside of carcass	89	46	52	(Kwantes and Isaac 1975)
U.K.	Frozen Chicken	Swab - inside and outside of carcass	64	41	64	(Kwantes and Isaac 1975)
U.K.	Mostly Frozen chicken	Swab of skin surface	68	10	15	(Gitter 1976)
U.S.A.	Fresh broilers	Not specified	40	17	43	(Bailey and Fletcher 1987)
Sweden	Fresh chicken	200 cm^2 of skin and meat - homogenized	45	0	0	(Ternstrom and Molin 1987)
Switzerland	Fresh chicken	Not specified	56	10	18	(Breer and Schopfer 1988)
New Zealand	Retail poultry portions	25-100g samples	25	12	48	(Lowry and Tiong 1988)
U.K.	Fresh chickens	Composite of skin and meat	50	33	66	(Pini and Gilbert 1988)
U.K.	Frozen chicken	Composite of skin and meat	50	27	54	(Pini and Gilbert 1988)
Denmark	Neck skin	2-3 g of neck skin - Stomacher	17	8	47	(Skovgaard and Morgen 1988)
U.S.A.	Fresh broilers	Carcass rinse	90	21	23	(Bailey et al. 1989)

Table 7 - Continued

Origin	Type of Poultry Product	Type of Sample Taken	No. of Samp.	No. Pos.	% Pos.	Reference
Canada	Retail chicken legs	25 g of sample into enrichment broth	16	9	56.3	(Farber et al. 1989)
U.K.	Fresh chicken at a proc. plant	10 g of neck skin - homogenized	30	15	50	(Hudson and Mead 1989)
U.S.A.	Fresh chicken wings	Skin portion removed and rinsed	50	5	10	(Genigeorgis et al. 1989)
U.S.A.	Fresh chicken drumsticks	Skin portion removed and rinsed	60	14	23	(Genigeorgis et al. 1989)
U.S.A.	Fresh chicken livers	Livers were rinsed	50	7	14	(Genigeorgis et al. 1989)
U.S.A.	Fresh turkey wings	Skin portions removed and rinsed	60	12	20	(Genigeorgis et al. 1990)
U.S.A.	Fresh turkey drumsticks	Skin removed and rinsed	60	8	13.3	(Genigeorgis et al. 1990)
U.S.A.	Fresh turkey tails	Tails were rinsed	60	7	11.7	(Genigeorgis et al. 1990)
U.K.	Precooked/chilled retail chicken	25 g sample - homogenized	102	27	26.5	(Kerr et al. 1990)
U.K.	Processed chicken at plant	Skin samples and swab samples	16	10	62.5	(Mead et al. 1990)
Austra-lia	Frozen chickens	Rinse sample	80	12	15.0	(Varabioff 1990)
Austra-lia	Fresh chicken at plant	Rinse samples	12	1	8.3	(Varabioff 1990)
Taiwan	Retail chicken carcass	25 g sample - blended	16	8	50	(Wong et al. 1990)
U.K.	Retail chicken carcasses	10 g of neck skin	25	14	56	(Lewis and Corry 1991)
Norway	Processed chicken at plant	Rinse sample	90	55	61	(Rorvik and Yndestad 1991)
Sweden	Processed broilers at plant	Neck-skin from 5 broilers pooled	136	36	26.5	(Loncarevic et al. 1994)
England	Retail raw chicken	25 g sample - homogenized	32	21	65.6	(MacGowan et al. 1994)

2.4.4. CONCENTRATION OF *LISTERIA* SPP. ON POULTRY PRODUCTS

Few workers who have investigated the incidence of *Listeria* spp. on poultry products have determined the concentration of the bacteria on meat or in the processing plant. One report from the U.K. indicated that samples of cooked poultry meat carried less than 100 *L. monocytogenes* CFU/g, while levels as high as 1000 CFU/g have been reported for fresh red meat (Genigeorgis et al. 1989). Recently, the concentration of *Listeria* species on a number of meat products was determined. The results are presented in Table 8.

Table 8 - Concentration of *Listeria* spp. in Poultry Products

Specimen	*Listeria* spp. / gram	Species Identified
Chicken quarter (raw)	850	*monocytogenes* + *innocua*
Chicken gougons	1200	*monocytogenes* + *innocua*
Vegetable crisp bakes	200	*monocytogenes*
Cornish lamb chop (raw)	50	*innocua*
Chicken quarters (raw)	500	*monocytogenes* + *innocua*
Chicken thighs (raw)	100	*monocytogenes*

Source: (MacGowan et al. 1994)

2.5. Chemistry of Ozone in Aqueous Solutions

Ozone is a light blue gas at room temperature that is generated by passing electricity through air and converting oxygen (O_2) to ozone (O_3). Ozone's thermodynamic properties are such that it cannot be liquefied by compression. Concentrating ozone in liquid oxygen is relatively safe up to 30 percent by weight, but spontaneous explosions occur at concentrations of more than 72 percent by weight (Bablon et al. 1991b). Since ozone decomposes even when dissolved in a liquid, it must be generated onsite for water treatment purposes.

Oxidation reactions initiated by ozone in water are complex. In aqueous solution, ozone may act on various compounds in one of two ways: a) by direct reaction with the molecular ozone, and b) by indirect reactions by means of radical species that are formed during ozone decomposition (Hoigne and Bader 1978). In ground and surface waters used as sources for drinking water (pH 7-8) the two

reaction pathways can be of comparable importance (Hewes and Davison 1971; Hoigne and Bader 1978).

2.5.1. DIRECT REACTIONS IN WATER

Direct reactions take place when organic and inorganic compounds are oxidized by ozone molecules. These reactions tend to be highly selective and are quite often slow (minutes) (Hoigne and Bader 1978). Rate constants of direct reactions of ozone with non-dissociating organic compounds have been determined from the absolute rates with which ozone reacts in the presence of various concentrations of these compounds in water (Hoigne and Bader 1983a). Compounds studied included aliphatic alcohols, olefins, chlorosubstituted ethylenes, substituted benzenes and carbohydrates. The kinetics of all the reactions studied were first order with respect to ozone and solute concentration.

Similar work was carried out to determine direct reaction rate constants for ozone and dissociating organic compounds such as amines, amino acids, carboxylic acids and phenols (Hoigne and Bader 1983b). Reaction rates of ozone with 40 inorganic aqueous solutes including compounds of sulfur, chlorine, bromine, nitrogen and oxygen, as well as free radicals have been determined (Hoigne et al. 1985). All of the individual direct reactions in water were first order with respect to substrate and ozone concentrations.

Ozonation byproducts of direct reactions include aldehydes, formaldehyde, heptanal, ketones, acids, linear alkanes, substituted aromatics, alkanals, carboxyclic acids, brominated byproducts and alkanoic acid esters (Bablon et al. 1991b; Ferguson et al. 1990; Glaze et al. 1989; Killops 1986). Half of the compounds present in solution prior to ozonation that are identified remain unaffected by ozonation (Killops 1986).

2.5.2. INDIRECT REACTIONS IN WATER

Indirect reactions take place when aqueous ozone decomposes in a series of reactions in which intermediate radical species are produced (Hoigne and Bader 1978). The highly reactive hydroxyl radical is believed to be the most important decomposition product having a life span of only a few microseconds in water (Finch et al. 1992). Radicals formed in the decomposition process can oxidize solutes or additionally accelerate the decomposition of ozone (Staehelin and Hoigne 1982).

Three types of chemical compounds (initiators, promoters, and inhibitors) are thought to be involved in ozone decomposition (Bablon et al. 1991b). Initiators are

compounds that can react with ozone and produce a superoxide ion (O_2^-) as a product which will continue to react with ozone. Examples of initiators include inorganic compounds, hydroperoxide ions (HO_2^-) and some cations. Promoters, such as phosphate species, formic acid, primary alcohols and humic acids, are organic and inorganic molecules that can react with the hydroxyl radical (OH^-) to produce O_2^- which reacts much more readily with ozone than with other aqueous solutes (Bablon et al. 1991b). Inhibitors are defined as being compounds that consume OH radicals without regenerating O_2^- and form relatively inert products. Common inhibitors include bicarbonate and carbonate ions, tertiary alcohols and humic substances (Hoigne et al. 1985).

Two major models (having 9 or 10 reaction steps) have been proposed to describe ozone decomposition chain reactions; namely, the Hoigne, Staehelin and Bader model; and the Gordon, Tomiyasu and Fukutomi model (Bablon et al. 1991b). The Hoigne, Staehelin and Bader model proposes that the initiation step is characterized by an oxygen radical transfer from the ozone molecule to the hydroxide radical, and some of the subsequent chain reactions are thought to involve the intermediates HO_3 and HO_4. The initiation step in the Gordon, Tomiyasu and Fukutomi model is thought to involve a two-electron transfer or an oxygen atom transfer from ozone to the hydroxide ion and the proposed intermediate species do not include HO_3 and HO_4.

Decomposition kinetics of ozone in aqueous solutions have been studied in which pseudo-*n*-order kinetic models were fitted to the observed decomposition data, where *n* may be either first, three-halves, or second order (or a combination of first and second order terms) (Gurol and Singer 1982; Hewes and Davison 1971; Peleg 1976; Staehelin and Hoigne 1982; Teramoto et al. 1981; Tomiyasu et al. 1985). However, recently it has been shown that pseudo-*n*-order kinetics are inappropriate because they generally do not provide for changes in the reactive character of the solute (Watt et al. 1989). Ozone consumption rates appear to depend on how much ozone has already been consumed, as well as on the instantaneous ozone concentration. Replacement of the pseudo-*n*-order constant *k* with a variable (*w*) representing the specific ozone utilization rate, showed that the behavior of the variable was dependent on sample character, the consumed ozone dose and the dilution factor used in the rate test (Watt et al. 1989). In every case, values of *w* decreased as ozone was consumed indicating that reactive compounds were being oxidized to less reactive species.

Ozone decomposition in water is influenced by a number of variables including pH, temperature, the organic and inorganic constituents of the water, and the presence of inorganic and organic initiators (Finch et al. 1992). The main parameters affecting the decomposition rate in pure aqueous systems are temperature and pH (Sullivan and Roth 1979). Ozone decomposition increases as the temperature of the solution increases and as the pH increases (Bablon et al. 1991b; Sullivan and Roth 1979). It has been reported that decomposition is relatively slow at pH values less than 6, while decomposition becomes significant for pH values ranging from 6 to 9.5 (Gurol and Singer 1982). Decomposition of ozone at pH values less than 4 appear to be independent of pH (Hewes and Davison 1971). In addition, the rate of decomposition may be reduced in the presence of radical scavengers such as carbonate and bicarbonate ions (Dore et al. 1987).

2.6. Effect of Water Quality Parameters on Ozone Disinfection

Water quality parameters may influence ozone disinfection results by either creating an ozone demand, decomposing ozone at a faster rate, or physically protecting the microbes from ozonation (Helmer 1992). Significant water quality parameters include temperature, pH, turbidity, and alkalinity. These parameters are discussed with respect to surface water quality, although they also would bear consideration in dealing with poultry chiller water. The last section deals with characteristics of poultry chiller water that would likely have an impact on ozone disinfection in this media.

2.6.1. TEMPERATURE

Although it has been claimed that ozone inactivation of microorganisms generally increases at higher temperatures (Bablon et al. 1991a), this is difficult to justify in the light of what has been reported in the literature. While it is true that some organisms appear to be more easily inactivated at higher temperatures, other workers have reported that some organisms appear to be unaffected by temperature changes, while other organisms are more easily inactivated by ozone at colder temperatures.

The resistance of both *Naegleria gruberi* and *Giardia muris* cysts to ozone decreased with increasing temperatures (Wickramanayake et al. 1984b). *N. gruberi* was about five times more resistant than *G. muris* at 25 °C, but only two times more

resistant at 5 °C. The change in resistance to ozone with temperature appears to vary depending on the organism. Similar results were obtained from ozonation of *Giardia lamblia* cysts where the resistance of the cysts to ozone inactivation at 5 °C was nearly three times greater than that at 25 °C (Wickramanayake et al. 1984a). Six enteroviruses were less resistant to ozone disinfection when the temperature of the suspending medium was raised from 5 °C to 10 °C (Roy et al. 1982).

Greater levels of inactivation were observed for ozonation of *Mycobacterium fortuitum* at higher temperatures with the same applied ozone dose, even though the residual ozone levels were lower at the higher temperatures (Farooq et al. 1977b). In the case of *Cryptosporidium parvum*, ozonation at warmer temperatures (22 °C) gave a higher degree of inactivation than was observed for experiments at 7 °C (Finch et al. 1993a).

In another experimental design, a reaction vessel was fed with constant streams of ozone gas and organism suspension so that a constant concentration of ozone was maintained both for experiments at 20 °C and at 10 °C in the reaction vessel (Herbold et al. 1989). Experimental conditions other than temperature were held the same. A 10^4 reduction of hepatitis A virus was achieved at 10 °C in roughly one quarter of the time it took at 20 °C. Poliovirus 1 and *E. coli* were also much more rapidly inactivated at 10 °C than at 20 °C.

When fresh chicken meat was ground up, diluted with water and filtered to obtain a fresh meat microbial suspension with an initial count of $10^{3.63}$ CFU/mL, microbes did not survive ozone treatment for 10 min at 2 °C under a flow rate of 650 mL/min (Yang and Chen 1979b). Under the same conditions at 25 °C, there was only about a 0.5 log reduction in microbial numbers.

In contrast to these findings, the effect of temperature on ozone inactivation of MS2 coliphage and HPC bacteria using a factorial experimental design demonstrated that lowering the temperature from 22 °C to 4 °C did not have a significant effect on the inactivation results (Helmer and Finch 1993). The inactivation rates for poliovirus 1 remained the same when the temperature was raised from 10 °C to 20 °C (Herbold et al. 1989).

2.6.2. pH

The impact of pH on the bactericidal effects of ozone seems minor. The variation in disinfection efficiency with different pH values appears to be due to

changes in the ozone decomposition rate because ozone decomposes faster in aqueous solutions with a high pH (Bablon et al. 1991a).

Several studies have reported that pH was not a significant factor in ozone disinfection. Inactivation of hepatitis A virus, simian rotavirus and human rotavirus did not appear to be related to changes in pH values between 6 and 8 (Vaughn et al. 1987; Vaughn et al. 1990).

Inactivation trends have not been consistent among organisms that are resistant to ozone when they are ozonated at several pH levels. *N. gruberi* cysts were more resistant to ozone at pH 9 but there was little variation in the organism's resistance to ozone at pH values ranging from 5-8 (Wickramanayake et al. 1984b). The opposite was the case for *Giardia muris* which was less resistant to ozone at pH 9 than at pH 5-7 (Wickramanayake et al. 1984b). A study with a factorial design investigated the effects of several water quality parameters on ozone disinfection of *G. muris* including experiments conducted with pH values of 5.7 and 7.6 (Labatiuk et al. 1992). For contact times ranging from 2 to 7 minutes, pH was a significant factor ($P<0.05$). The survival ratios for *M. fortuitum* increased as the pH was increased from 5.7 to 7.0 to 8.5 to 10.1 even though the ozone residual decreased (Farooq et al. 1977a). Similarly, the resistance of two viral strains was greater at pH 4.3 than at pH 7.2 (Roy et al. 1982).

A suspension of spoilage organisms was obtained by rinsing spoiled chicken gizzards (Yang and Chen 1979b). Samples of the suspension were adjusted to pH values of 3, 5, 7, 9, and 11 and ozonated at 2 °C and 25 °C. The results are summarized in Table 9. Greater microbial inactivation was observed as the pH was decreased. Regardless of the pH value, the bactericidal effect of ozone was greater at 2 °C than that at 25 °C. At a given temperature, the decrease in log reduction with increase in pH is most likely due to greater ozone decomposition associated with increasing pH. No differences were apparent in the log reductions obtained for pH values of 5, 7, and 9 at a given temperature.

2.6.3. TURBIDITY

In natural waters microorganisms are not generally in the free state but tend to be fixed on the surface of mineral or organic matter, incorporated with human, animal, or cell debris, or associated with flocs (Bablon et al. 1991a).

No protective effect was observed for ozonation of poliovirus (Sabin Type 1) adsorbed by clay particles (5 turbidity units -TU) when the results were compared with

unadsorbed poliovirus suspensions (Boyce et al. 1981). Similar results were observed for adsorbed coxsackievirus A9. Although *E. coli* did not necessarily adsorb to bentonite clay particles, similar inactivation data were obtained for controls and suspensions containing bentonite clay particles.

Table 9 - Effect of Temperature, pH and Ozone Log_{10} Reductions on Total Microbial Counts

Temperature	Log_{10} Reduction at Various pH Values				
	3	5	7	9	11
2 °C	4.74	3.25	3.17	3.22	1.25
25 °C	3.78	1.41	1.68	1.25	0.30

Source: (Yang and Chen 1979b)

Similar experiments to those just described were conducted with the same organisms using aluminum hydroxide to adsorb poliovirus (Sabin Type 1), coxsackievirus A9 and *E. coli* (Walsh et al. 1980). None of these organisms were afforded protection by 5 TU of floc particles.

However, when poliovirus and coxsackievirus were associated with human epithelial cells to simulate cells which may be sloughed from the intestinal tract of infected individuals, a significant level of protection against ozone inactivation was observed (Emerson et al. 1982). In the case of poliovirus, unassociated poliovirus was completely inactivated at an applied ozone concentration of 0.28 mg/L while an applied ozone dose of 4.06 mg/L with a 40 second residual ozone concentration of 2.56 mg/L did not totally inactivate the cell-associated poliovirus. Associated coxsackievirus was more difficult to inactivate than poliovirus.

When poliovirus were associated with fecal matter, unadsorbed poliovirus were completely inactivated within 10 seconds at an ozone concentration of 0.012 mg/L (Foster et al. 1980). Similar exposure of fecal-associated viruses to ozone did not result in its complete inactivation indicating that some level of protection was afforded by the fecal matter.

2.6.4. ALKALINITY

Alkalinity in the form of bicarbonate and carbonate ions may be important to the inactivation of microbes because they act as inhibitors to ozone decomposition by reacting with intermediate free radicals. This means that more ozone will become available for direct more selective reactions and that less OH radical-induced oxidation

will occur (Bablon et al. 1991b). Bicarbonate and carbonate ions can extend the life of ozone in water but at the same time it is most likely that they are going to have a negative effect on the oxidation rates of organic compounds (Dore et al. 1987). Removal of carbonate ions from groundwater (pH 7.7) reduced the half-life of ozone from 20 to 2 minutes even when the pH was kept constant by adding a buffer (Hoigne 1994).

Ozone disinfection of *E. coli* in phosphate and carbonate-phosphate buffers showed that alkalinity significantly inhibited ozone disinfection of *E. coli* in ozone demand free water (Finch et al. 1992). Because alkalinity scavenges intermediate free radicals, direct reactions were responsible for the inactivation of the bacteria.

2.6.5. POULTRY CHILLER WATER PARAMETERS

One of the steps in poultry processing plants involves the rapid cooling of dressed birds to remove body heat. This is accomplished by immersing the birds in water at a temperature ranging from 0° to 5 °C. Some plants have a single chiller, while other plants have a prechiller unit that partially cools the poultry carcasses and the rest of the cooling process takes place in the main chiller unit. Overflow water from prechiller and chiller tanks contains both dissolved solids in the form of soluble proteins and salts and suspended solids in the form of grease and colloidal protein (Chang et al. 1989). The actual values measured for any given chiller water parameter will vary from plant to plant. Some representative values for prechiller water BOD, COD, pH, total solids, volatile suspended solids, FOG and ammonia (as nitrogen) are presented in Table 10, while Table 11 shows representative values for chiller water.

Table 10 - Spent Prechiller Water Characteristics

BOD mg/L	COD mg/L	pH	TS mg/L	VSS mg/L	FOG mg/L	NH_3-N mg/L	Source
---	843	6.88	675	---	234		(Chang and Sheldon 1989)
442	692	---	776	523	800	---	(Crosswhite et al. 1971)
780	---	---	---	---	---	---	(Dart 1974)
956	1193	7.1	1411	985	270	28	(Hamza et al. 1978)

BOD - biochemical oxygen demand, COD - chemical oxygen demand, TS - total solids, VSS - volatile suspended solids, FOG - fats/oil/grease, NH_3-N - ammonia as nitrogen

Table 11 - Spent Chiller Water Characteristics

BOD mg/L	COD mg/L	pH	TS mg/L	VSS mg/L	FOG mg/L	NH_3-N mg/L	Source
320	435	---	514	331	250	---	(Crosswhite et al. 1971)
---	903	---	705	549	165	---	(Hamm 1972)
860	1104	---	1135	---	---	---	(Layton 1972)
---	903	---	---	---	165	---	(Carawan et al. 1974)
790	---	---	---	---	---	---	(Dart 1974)
758	1193	7.4	1093	644	239	29	(Hamza et al. 1978)
1250	---	---	---	---	---	---	(Gelman 1987)

BOD - biochemical oxygen demand, COD - chemical oxygen demand, TS - total solids, VSS - volatile suspended solids, FOG - fats/oil/grease, NH_3-N - ammonia as nitrogen

In addition to the chiller water characteristics listed in Tables 10 and 11, another component of chiller water is microorganisms that are introduced into poultry chiller water by chicken carcasses as they are cooled. The concentration of coliforms in chiller water at one plant averaged 2.1×10^3 CFU/mL in the prechiller and 1.3×10^3 CFU/mL in the chiller (Layton 1972). Total aerobic plate counts were higher, averaging 6.9×10^4 CFU/mL in the prechiller and 6.9×10^4 CFU/mL in the chiller (Layton 1972). In another plant the concentration of coliforms and *E. coli* in spent chiller water were each 2.4 MPN/mL (Sheldon and Chang 1987). The average concentration of *Campylobacter jejuni* in 21 chiller water samples that were positive for the organism was 354 CFU/mL (range was from 19 to 4.9×10^5 CFU/mL) (Genigeorgis et al. 1986).

Although the use of ozone to recondition chiller waters has been investigated (Chang and Sheldon 1989; Sheldon and Chang 1987), it does not appear as though any work has been conducted to determine the decomposition of ozone in chiller water. After ozonation of chiller water for 10 minutes, FOG, COD, and TS were reduced 76, 48 and 19% respectively. After 50 minutes of ozonation the chiller water was clear, colorless, and essentially free of grease. Examination of the foam composition that rose to the top of the reactor indicated the presence of proteins and lipids. The pH decreased over the 50 minutes from 6.88 to 5.60 and this was attributed to the oxidation of lipids and their associated aldehydes as well as the oxidation and potential hydrolytic destruction of proteins (Chang and Sheldon 1989).

The use of ozone as a disinfectant during chilling of poultry meat is discussed below in Section 2.7.4. Here it is sufficient to note that high concentrations of organic matter in chiller water causes increased ozone demands that reduce the effectiveness of ozone as a disinfectant (Chang and Sheldon 1989).

2.7. Inactivation of Bacteria with Ozone

A search has been going on for an ideal bactericidal agent that will eliminate bacteria from fresh processed poultry meat without adversely affecting the finished product. The bactericidal properties of a large number of chemicals and gases have been evaluated. Some of the treatments that have demonstrated the greatest success for eliminating bacteria have either had an adverse effect on the appearance of the finished product, produced offensive odors, or are not approved for use on food products. So the search for an economic yet potent disinfectant for meat products continues. Ozone has demonstrated potential as a powerful bactericidal agent.

2.7.1. INACTIVATION MECHANISM AND CHEMICAL REACTION PATHWAY

The mode of action of ozone on microorganisms is poorly understood. Some have suggested that the primary attack of ozone was on the cell wall or membrane of the bacteria (probably by reaction with the double bonds of lipids) and that lysis of the cells depends on the extent of that reaction (Hamelin and Chung 1974; Scott and Lesher 1963). Others have proposed that oxidation of unsaturated fatty acids in the membrane led to the loss of membrane function, but this was shown not to be the case where an *Escherichia coli* strain unable to synthesize unsaturated fatty acids was no less resistant to ozone than strains capable of synthesizing unsaturated fatty acids (Ohlrogge and Kernan 1983). Others have suggested that ozone penetrates cell membranes and damages chromosomal DNA which may be one of the factors responsible for the death of the cell (Ishizaki et al. 1987).

Evidence is mounting that the direct reaction pathway is the most important one influencing disinfection rather than the radical reaction pathway although some researchers have proposed that the radical mechanism was the most important (Bancroft et al. 1984; Dahi 1976). One argument against the radical pathway is that reactions involving radicals are so fast that they could be consumed before they encountered a microorganism, but depending on the solutes present, some intermediate products may have a long enough life to play a part in disinfection

(Hoigne and Bader 1978). Support for the direct reaction pathway comes from studies using ozone and hydrogen peroxide for disinfection (Wolfe et al. 1989a; Wolfe et al. 1989b). Disinfection was less effective at higher ratios of peroxide to ozone which was thought to be due to a simultaneous lower ozone residual caused by these experimental conditions.

2.7.2. EFFECT OF OZONE ON MICROORGANISMS RELEVANT TO POULTRY PROCESSING

After propagating *Staphylococcus aureus, Salmonella typhimurium*, enteropathogenic *Escherichia coli* **O**126:**B**16 and *Pseudomonas fluorescens* in Trypticase soy broth or Penassay broth for 18 hours at appropriate temperatures, the cultures were centrifuged for 15 minutes at 1,000 x *g*, and the resulting pellet of organisms was suspended in phosphate-buffered saline (PBS) with a final volume of 1000 mL (Burleson et al. 1975). Ozone was added through a porous diffuser placed at the bottom of a plexiglass treatment column at a flow rate of 152.4 cm^3 per hour. The treatment column had a volume of 1,700 mL. Residual ozone levels were assayed in 1% potassium iodide in neutral phosphate buffer and determined by spectrophotometric absorbance at 352 nm. *S. aureus, S. typhimurium, E. coli* **O**126:**B**16, and *P. fluorescens* were not detected after 15 seconds of treatment with ozone which was the first time interval at which data was collected.

Escherichia coli

The kinetics of ozone inactivation of *E. coli* have been investigated (Finch et al. 1988). The disinfection reaction of *E. coli* in ozone demand-free phosphate buffer was not first-order with respect to the surviving bacteria concentration. Two distinct stages were observed: there was an initial rapid inactivation stage followed by a slower inactivation stage. The dose-response relationship between ozone and *E. coli* had a "tail" where the first 3.5 log units of *E. coli* removal only consumed a small amount of ozone. However, a 7 log unit reduction in *E. coli* required 45 times more ozone than the amount required for a 3.5 log unit reduction. The "tail" effect in the ozone-dose curve results in increasingly larger doses of ozone being required to remove increasingly fewer bacteria for each increment.

Ozone inactivation of *E. coli* (ATCC 11775) in water designed to delay ozone decomposition was compared to inactivation in water that was designed to rapidly deplete ozone (Finch et al. 1992). A stock ozone solution with a concentration of 13 mg/L at 22 °C was used. The inactivation rate of *E coli* increased with longer contact

times in a phosphate/bicarbonate buffer designed to prolong the life of dissolved ozone. Only 6 seconds of contact time was required to achieve a 3.7 log inactivation, but at 600 seconds there was close to a 7 log inactivation. In a phosphate buffer containing hydrogen peroxide designed to rapidly decompose the ozone, a 6 second contact time gave a 3.8 log inactivation. After 120 seconds of contact there was a 4.3 to 5 log inactivation, after which increasing the contact time did not give an increase in inactivation. This was due to the fact that after 60 seconds in this arrangement the residual ozone concentration was below detectable levels. These results demonstrate that it is important to obtain an understanding of the chemical composition of the water containing microorganisms to be inactivated with ozone. If the water promotes rapid decomposition of ozone, effective disinfection will be more difficult to achieve.

Pseudomonads

Broiler necks were allowed to spoil and then were rinsed to make up a suspension of spoilage organisms (Yang and Chen 1979a). The microbial suspension was treated with an gaseous ozone concentration of 2.48 mg O_3/L for 9 min at a flow rate of 3175 mL/min and an identical control was treated with air instead of ozone. The initial total microbial count of $10^{8.3}$ org./mL was reduced to $10^{4.95}$ org./mL and the MPN of coliforms was reduced from $10^{4.97}$ CFU/mL to $10^{2.63}$ CFU/mL. A total of 119 cultures were obtained with 60 cultures from the ozone treated samples and 59 cultures from the control samples. Ozone treated samples had 52.7% gram-positive cocci and 12.7% gram-negative cocci. Air treated control samples had 39.6% gram-positive cocci and 22.4% gram-negative rods. It could be inferred that ozone was more effective against gram-negative rod-type organisms than any of the other types.

Bacillus cereus

B. cereus cells were grown in tryptic soy broth at 30 °C, centrifuged and suspended in water at concentrations close to 10^6/mL for disinfection experiments (Broadwater et al. 1973). *B. cereus* spores were harvested from a sporulation medium that had been agitated while incubating at least 12 hours in a water bath at 30 °C. Suspensions were subjected to a range of dissolved ozone concentrations. *B. cereus* spores were 10 to 15 times more resistant to ozone than vegetative cells.

Six strains of *Bacillus* spores were conditioned at 54% relative humidity (Ishizaki et al. 1986). Spore samples were placed on strips of filter paper and subjected to a gaseous ozone concentration of 3 mg/L at a selected range of relative humidities. A relative humidity of 80% or more was required to attain significant sporicidal activity. Five strains of *Bacillus* spores exhibited a lag phase in which there

was no death for 0.5 to 2 hours (depending on the relative humidity) after which the inactivation rates were rapid at higher humidities. *B. cereus* spores were different than spores of other species in that inactivation began as soon as the sample was exposed to ozone. The D-value at 95% relative humidity was 15 minutes. (The D value is the time in which 90% of the population of bacteria at a given temperature will be killed). At 80% relative humidity the D value increased to 42 minutes, while at 70% relative humidity it was 123 minutes. Spores exposed to ozone on glass fiber filters were more resistant than spores on filter paper.

2.7.3. RELATIVE RESISTANCE OF MICROORGANISMS TO DISINFECTION BY OZONE

Several researchers have carried out experiments in which several organisms were subjected to the same ozonation protocol allowing their relative resistance to ozone to be determined. However, an overall ranking of the resistance of microorganisms to ozone disinfection from the literature is difficult because of the variety of experimental methods and ozone protocols that have been used.

The relative resistance of several microorganisms to inactivation by ozone has been reviewed (Bablon et al. 1991a). *E. coli* is one of the most sensitive bacteria while Gram-positive cocci (*Staphylococcus* and *Streptococcus*), the Gram-positive bacilli (*Bacillus*), and the *Mycobacteria* are most resistant. Viruses are generally more resistant to ozone than vegetative cells of bacteria but not more than *Mycobacteria*. Protozoan cysts are much more resistant than vegetative forms of bacteria and viruses. *G. lamblia* cysts have a sensitivity to ozone equivalent to sporular forms of *Mycobacteria* while *Naegleria* cysts are much more resistant than *Giardia* cysts. *Cryptosporidium* oocysts appear to be one of the most resistant organisms.

S. aureus, *S. typhimurium*, *E. coli*, *P. aeruginosa*, *P. fluorescens*, and *P. putida* were cultured in a nutrient broth and diluted using sterile phosphate buffered solution (Chen et al. 1992). A portion of the bacterial suspension was poured into 4 L of 0.8% saline to obtain a final cell concentration of 10^5-10^7 CFU/mL for each of the organisms. The saline solution containing bacteria was then flushed with ozone at a flow rate of 100 mL/min. Samples were collected after 1 minute and then at 2 minutes intervals. One loopful of ozonated suspension was immediately transferred to 10 tubes containing 5 mL of nutrient broth. The percent survival of each bacterial strain after treatment was determined from the ratio of the number of tubes indicating growth to the number of tubes tested. The residual ozone concentration was determined using

the idometric titration method. The results from this study are presented in Table 12. The most sensitive organism under these experimental conditions was *S. aureus* followed by *E. coli* and *P. aeruginosa*. The most resistant strain was *S. typhimurium*. While *E. coli* appeared to be more resistant than *S. aureus* to ozone in saline solution, *S. aureus* appeared to be more resistant than *E. coli* when suspended in secondary effluent (Burleson et al. 1975; Chen et al. 1992).

Table 12 - Bactericidal Effect of Ozone on Microorganisms Suspended in 0.8% Saline at 25 °C [a]

Microorganism	Inhibition time (min)[b]	Final ozone concentration showing inhibition (mg/L)
Salmonella typhimurium	17	0.54
Pseudomonas fluorescens	13	1.07
P. putida	11	0.78
P. aeruginosa	9	0.34
Escherichia coli	9	0.50
Staphylococcus aureus	5	0.30

[a] Microorganisms were seeded to 10^5-10^7 CFU/mL suspension, prior to ozonating.
[b] Time needed to show 100% inhibition obtained from the ratio of the number of tubes indicating no growth to the number of tubes tests (10 in this test)

Source: (Chen et al. 1992)

At 20 °C, the sensitivity of two virus strains and three species of bacteria to ozone was determined (Herbold et al. 1989). The results are listed here in order of increasing resistance to ozone: poliovirus 1 < *E. coli* < hepatitis A virus < *Legionella pneumophila* < *Bacillus subtilis* spores.

Individual experiments where approximately 3 x 10^7 CFU/mL of *S. aureus*, *S. typhimurium*, enteropathogenic *E. coli* **O**126:**B**16, and *P. fluorescens* were suspended in secondary effluent from a wastewater treatment plant and exposed to ozone (flow rate 2.54 mL/min) in a column reactor (Burleson et al. 1975). Complete inactivation of the most resistant organism (*S. aureus*) under these conditions took 2 minutes. *P. fluorescens* was completely inactivated in 1.5 minutes, while *S. typhimurium* and *E. coli* **O**126:**B**16 were completely inactivated in 1.25 minutes. *B. cereus* was slightly less resistant than *E. coli*, while *B. cereus* spores were significantly more resistant than *E coli*.(Broadwater et al. 1973).

A study of the comparative resistance of HPC bacteria, *E. coli* and MS2 coliphage to ozone and PEROXONE disinfection showed that the HPC bacteria were considerably more resistant than *E. coli* or MS2 coliphage (Wolfe et al. 1989a). A more recent comparison of the resistance of HPC bacteria and MS2 coliphage demonstrated that MS2 coliphage was more sensitive to ozone (Helmer and Finch 1993). Experiments with *G. muris* and R2A HPC bacteria showed that they were somewhat comparable in their resistance to ozone and PEROXONE (Scott et al. 1992). Other workers found that *G. muris* was slightly more sensitive to ozone than HPC bacteria (Helmer and Finch 1993).

The relative resistance of microorganisms to ozone is summarized in Figure 1 where the solid lines indicate known relationships. Dashed lines indicate contrary findings in the literature in which one organism may be more resistant than another under certain experimental conditions. Organisms on the same line that are connected by a solid line and arrows have the same relative resistance to ozone. Otherwise the relative resistance of organisms on the upper and lower part of the figure is difficult to ascertain from the literature because different ozone protocols were used by workers in each of the studies.

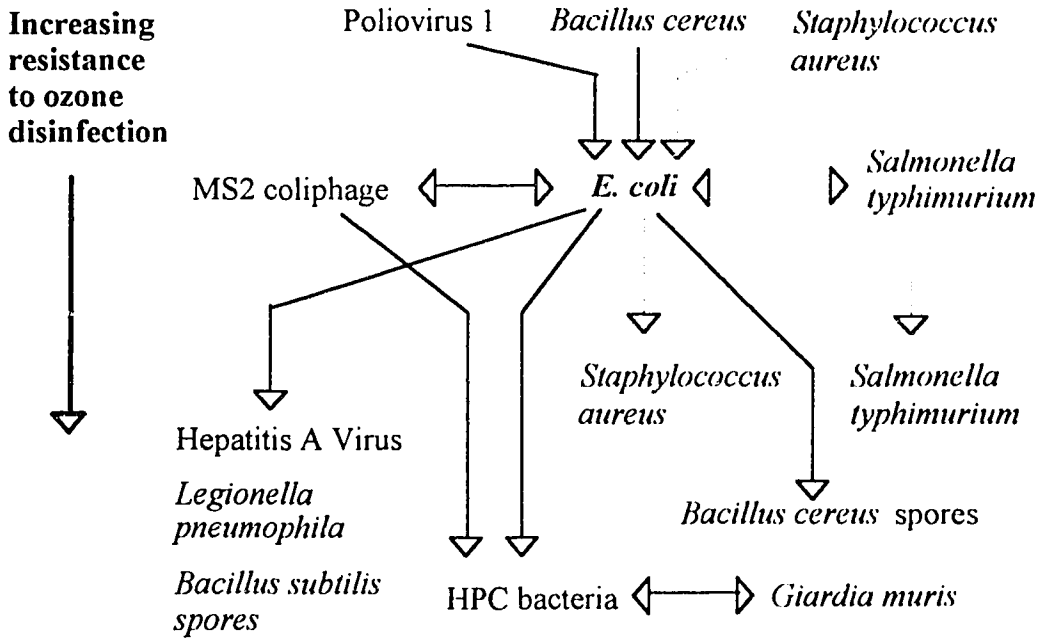

Figure 1 - Ranking of Selected Organisms' Resistance to Ozone Disinfection

2.7.4. USE OF OZONE AS A DISINFECTANT FOR POULTRY CARCASSES IN CHILLER WATER

Very few investigations into the use of ozone as a disinfectant for poultry carcasses during immersion chilling have been made. Before the work done by Adams at the University of Arkansas (Adams 1990) and the work done by Sheldon at North Carolina State University (Sheldon and Brown 1986) are addressed, a controlled experiment will be reviewed.

Controlled Experiment with Ozone and Spoilage Organisms on Chicken Parts

Eight pieces of breast and thigh meat were soaked in ice cold water (Yang and Chen 1979a). Ozone gas at 3.88 mg/L under a flow rate of 2050 mL/min was dispersed through the broiler parts for 20 minutes. Eight pieces used for controls were subjected to the same conditions using compressed air instead of ozone. After treatment, broiler parts were drained and stored at 4-5 °C in polyethylene bags. Three replicates were made. Immediately after ozone treatment, total microbial counts at 37, 29 and 7 °C were reduced 90.5, 90.5 and 86.0%, accordingly, compared with air treated controls. Using 10^7 CFU/cm^2 as a spoilage criteria, an extension of 2.4 days in shelf-life was found for ozone treated broiler parts compared with air treated controls. This study clearly demonstrated that ozone treatment of broiler parts effectively reduced gram-negative microorganisms on the products. The ozone treatment, however, did not affect the multiplication of the surviving microorganisms. The growth curves for spoilage organisms on ozone treated broiler parts had an identical shape to the controls, it was just shifted down a bit due to the 2.4 d extension of shelf life before spoilage occurred.

Experience with Ozone in a Giblet Chiller

The effects of ozonated chill water on microbiological and sensory characteristics of broiler carcasses and on the quality of chill water was investigated as part of the work in a master's thesis at the University of Arkansas (Adams 1990). A rotary giblet chiller with two separate compartments containing 400 liters of water was used. One half with 20 ppm chlorine was used as a control. The other half was used for ozonation.

Water was drawn from the bottom of the one end of the ozone tank and pumped to a ozone gas/solution mixer. After mixing, the treated water was returned to the chill tank at the other end, thus forcing untreated water toward the suction

intake. Ozone was added in sufficient quantities to maintain an oxidation reduction potential (ORP) reading of 300-400 (no units are given).

Three times (at 36, 42, and 47 days of age), a flock of 500 broilers was exposed to *Salmonella typimurium* through drinking water containing 10^8 *S. typhimurium* / mL. The birds were processed at 49 days of age. Prior to the start of the experiment 100 carcasses were chilled in each side of the giblet chiller to increase the organic load. After ozone generation commenced, a further 100 carcasses were chilled on each side of the chiller.

The results obtained from this system were poor. Rinse samples were taken from 50 carcasses after both methods of chilling. Each of the two groups of carcasses had a *Salmonella* incidence rate of 58% (29 out of 50 birds were positive in each case). The level of *Salmonella* on ozone-treated carcasses averaged 580 MPN/100 mL, while the average level detected on the control carcasses was 310 MPN/100 mL.

In addition, levels of total organisms and presumptive coliforms were significantly higher on the ozonated side of the chiller than they were on the side treated with 10 ppm chlorine. Adams suggested that either insufficient ozone was incorporated into the chiller or that the ozone produced was not remaining in solution long enough to have any beneficial effect, or perhaps agitation in the chiller brought the ozone to the surface where it rapidly dissociated. The fact that insufficient ozone was dispersed in the chiller was confirmed by analysis of water samples collected as water exited the ozone gas/solution mixer (this was located near where the ozone gas was introduced to the chiller). At this sampling point there was a 70 to 99% reduction in total organisms and presumptive coliforms.

The shelf life of birds from both types of chilling was similar. Levels of total organisms increased with time and on both groups reached spoilage levels after 12 days of storage.

The major weakness in this study lies in the ozone protocol used. It did not give the researchers any idea of actual ozone residual concentrations in the giblet chiller. As a result it appears that the ozone residual concentration in the giblet chiller was very low throughout the experiment.

Experience with Ozone in a Bench Scale Chiller

Comparisons were carried out between plain water and ozonated water in a bench scale chiller (Sheldon and Brown 1986). The chiller water was preozonated to a concentration of 3.0-4.5 mg/L aqueous ozone. Ozone entered the bottom of the column through a stainless steel diffuser countercurrent to the water flow while the

experiment was conducted. Ozone gas exiting the top of the column was bubbled into water in a separate tank and the ozonated water was pumped into the chiller at a constant rate. After 25 minutes of chilling 2.1 mg/L of ozone could still be measured in the chiller water using the indigo trisulfonate method. Ice was added as needed to maintain the temperature in the chiller at 7 °C.

Poultry carcasses were obtained from a poultry processing plant just prior to the point in processing where they would have entered the chiller and were put on ice and transported to the laboratory. The carcasses were sampled before and after chilling and analysis was carried out for aerobic plate counts (APC), psychrotrophic plate counts (PPC), coliforms, fecal coliforms and *Salmonella*. The number of birds sampled was quite small, with two birds sampled for each of three experimental replicates. The results for each of the samples were averaged. Water chilling gave consistent reductions less than 1 log unit. Ozonated chilling gave an additonal small reduction that was also less than 1 log unit. The appearance and sensory attributes of the ozone chilled carcasses was not adversely affected by the treatment.

A shelf-life experiment was run for 11 days and at the end of that time the carcasses were not yet spoiled. At 11 days the ozone chilled carcasses had slightly lower APC (log 4.72/cm^2) and PPC (log 7.30/cm^2) than the APC (log 4.80/cm^2) and PPC (log 7.72/cm^2) for the water chilled carcasses (Sheldon and Brown 1986). The authors did not discuss whether the outcomes were statistically significant. It was noted that slightly reduced microbial counts for carcasses and large reductions of bacteria in chiller water were also obtained by other experimenters using chlorine (Thiessen et al. 1984) and chlorine dioxide (Lillard 1980).

Assessment

The results to date from efforts to disinfect poultry carcasses with ozonated chiller water have met with limited success. This is a bit surprising given the effectiveness of ozone as a disinfectant for bacteria in various suspensions and on several different surfaces. Ozone has also proved to be very effective in disinfecting and reconditioning poultry process waste waters (Sheldon and Carawan 1988; Sheldon and Chang 1987; Sheldon and Merka 1992). However, in the case of the experiment by Adams, it appears that insufficient attention was given to the decomposition of ozone in the chiller tank, resulting in concentrations of ozone that were too low to be effective. Although Sheldon and Brown (1986) measured 2.1 mg/L of ozone after 25 minutes of chilling, they did not evaluate what was happening at the interface between ozonated chiller water and the skin of the carcasses. Would a higher level of ozone be

sufficient to meet the ozone demand of the skin surface and still have enough disinfection capability to reduce the levels of microorganisms to a greater degree? Sheldon and Brown (1986) did not evaluate a range of ozone doses and had a very small sample size for their experiment. So it appears that based on the work done to date, a verdict on the effectiveness of ozone as a poultry carcass disinfectant during chilling cannot be made.

3. Materials and Methods

3.1. Materials

3.1.1. NUTRIENT AGAR

Nutrient agar was made by adding 11.5 g of Nutrient Agar (Difco Laboratories, Detroit, Michigan) to 0.5 L of Milli-Q water in a 1 L Erlynmeyer flask. The mixture was autoclaved for at least 20 minutes at 121 °C. The flask was taken from the autoclave and allowed to cool in an oven kept at a temperature sufficient to keep the agar from solidifying. Plates were poured in a laminar flow hood.

3.1.2. TRYPTIC SOY BROTH YEAST EXTRACT (TSBYE)

A nutrient solution for growing *L. innocua* was made by adding 30 g/L of Tryptic Soy Broth (Difco) powder and 6 g/L of Yeast Extract (Difco).

3.1.3. PEPTONE

A 0.1% solution of peptone (Difco) was used for all dilutions and membrane filtration requirements.

3.1.4. STERILE MATERIALS

Filter housings were sterilized by placing the housing in a paper bag which was autoclaved for a minimum of 20 minutes at 121 °C.

Wash bottle flasks used for membrane filtration were sterilized by placing aluminum foil over both apertures followed by autoclaving for a minimum of 20 minutes at 121 °C.

3.1.5. OZONE DEMAND FREE (ODF) GLASSWARE

All glassware that was used to expose the bacteria to ozone was first made ozone demand free (ODF) by completely filling the containers with water containing 15-20 mg/L of dissolved ozone and then capping them with aluminum foil. After a minimum contact time of 30 minutes, the water was poured out, the foil was replaced, and the containers were placed in an oven to dry.

ODF water used to calibrate the spectrophotometers was made by first bubbling ozone/oxygen gas through Milli-Q water for either 15 minutes or 10 min/L,

whichever was longer. After the container sat for 30 minutes, the water was boiled for 10 minutes, allowed to cool, and then stored in an ODF bottle.

3.1.6. PHOSPHATE BUFFER SOLUTION

Phosphate buffer used for experiments consisted of deionized water (obtained from a Milli-Q system - Millipore Corp. model OM-140 operated at a resistivity of at least 18 MΩ/cm), 8.448 g/L of disodium hydrogen orthophosphate (Na_2HPO_4), and 6.818 g/L of potassium dihydrogen orthophosphate (KH_2PO_4) (BDH Inc., AnalaR grade), resulting in a 0.11 M solution with a pH of 6.9. Phosphate buffer solutions were mixed in 4 liter batches in an Erlynmeyer flask. To make the solution ODF, an ozone/oxygen gas mixture was bubbled through the phosphate buffer for 40 minutes. The flask was then covered with aluminum foil and allowed to sit for a minimum of 30 minutes to allow the ozone demand reactions to go to completion. Following this, the contents of the flask were boiled for 10 minutes. After cooling, the phosphate buffer was stored in ODF containers.

3.2. Methods

3.2.1. GROWTH OF *L. INNOCUA* FOR EXPERIMENTS

Listeria innocua 33090 from the American Type Culture collection (ATCC) was obtained from Dr. Stiles in Food Science and Nutrition at the University of Alberta. *L. innocua* was inoculated from a nutrient agar plate into TSBYE (see 3.1.2) and grown for 48 hours at room temperature on a rotary shaker (New Brunswick Scientific Co. Inc.) set at 100 rpm. One mL of the 48 hour culture was subcultured into 250 mL of TSBYE and was grown for another 48 hours under identical conditions. *L. innocua* was propagated in 2 bottles containing TSBYE every 48 hours while experiments were being run. One bottle was used to make up the organism stock solution for experiments, while the other was used to transfer the organism to a new set of two bottles.

After the first 22 experiments had been completed a protocol change was necessary because a different centrifuge had to be used. At this time *L. innocua* was grown in test tubes containing 5 mL of TSBYE incubated at room temperature (22 °C) on a rotary shaker set at 100 rpm. The organism was inoculated into two test tubes at a time. One of the test tubes was used to provide the stock solution for experiments and the other was used to transfer the organism to the next set of test

tubes. An Eppendorf pipette with sterile tips was used to transfer 0.1 mL of the old bacterial suspension to the fresh 5 mL of TSBYE in the test tubes.

One of the preliminary tasks undertaken was to determine when *L. innocua* entered the stationary phase of the growth curve. After the organism had been cultured in TSBYE, it was then subcultured in TSBYE and samples were removed at approximately 12-hour intervals. The concentration of bacteria was determined using pour plates, and the results appear in Table 13. At the beginning of the incubation period the TSBYE solution was clear, but after 48 hours the solution had turned cloudy due to the growth of *L. innocua*.

Table 13 - *L. innocua* Growth over 48 Hour Period in 250 mL of TSBYE

Hours of Incubation at Room Temperature	Concentration (organisms/mL)
6	1.54×10^9
24	2.69×10^9
30	2.52×10^9
48	1.59×10^9

After the protocol change when test tubes were used instead of bottles, another determination of the growth curve was made and the results are presented in Table 14.

Table 14 - *L. innocua* Growth over 48 Hour Period in 5 mL of TSBYE in a Test Tube

Hours of Incubation at Room Temperature	Concentration (organisms/mL)
0	2.99×10^7
16	2.01×10^9
23	1.33×10^9
39	1.45×10^9
48	1.50×10^9

From the results in Tables 13 and 14 it is apparent that *L. innocua* entered the stationary stage of the growth curve after 16 to 24 hours. Bacteria stock solutions

used in experiments were made up after the organism have been incubated at room temperature for 40-44 hours.

3.2.2. PREPARATION OF *L. INNOCUA* STOCK SOLUTIONS FOR EXPERIMENTS

All work involved in washing the organisms prior to experiments was carried out in a bio-safety hood. For the first 22 experiments which were conducted at 22 °C, 250 mL of *L. innocua* culture was centrifuged at 1600 x *g* for 20 minutes in a Jouan centrifuge (Model CR 4-22). The resulting pellet was washed twice with approximately 125 mL of ODF phosphate buffer and then resuspended in 250 mL of ODF phosphate buffer. From this organism stock solution 0.1 mL was added to reactor vessels containing 250 mL of ODF phosphate buffer to give an initial concentration ranging from 5.53×10^5 to 1.34×10^6 CFU/mL.

For the remaining experiments where *L. innocua* was grown in test tubes, test tubes containing 5 mL of TSBYE and *L. innocua* were centrifuged at 1415 x *g* for 20 minutes in a Sorvall centrifuge (Model SPX). The resulting pellet was washed twice with 5 mL of ODF phosphate buffer (pH 6.9) and then resuspended in 5 mL of ODF phosphate buffer. From this organism stock solution, 0.1 to 0.27 mL were added to 250 mL of ODF phosphate buffer to give an initial concentration ranging from 1.75×10^5 to 1.76×10^6 CFU/mL.

For cold temperature experiments the temperature setting on the Sorvall centrifuge was adjusted so that it kept the test tubes close to 4 °C. In addition phosphate buffer used to wash the bacteria was kept in a refrigerator at 4 °C.

3.2.3. OZONE APPARATUS AND RESIDUAL MEASUREMENT

The ozone apparatus consisted of an extra dry oxygen gas cylinder that supplied the feed gas to a Welsbach laboratory ozonator (Model T-816). A concentrated ozone solution was obtained by bubbling ozone/oxygen gas through two 500 mL gas absorption flasks in series that each contained 400 mL of Milli-Q water for a minimum of 15 minutes. Stock ozone solution used for experiments was taken from the second flask.

The ozone concentration in aqueous stock solutions was measured by UV spectrophotometry (Spectronic 601 - Milton Roy Company) at 260 nm with a molar absorptic coefficient of 3,300 M^{-1} cm^{-1}. This value of 3,300 M^{-1} cm^{-1} for the absorption coefficient is midway between the reported extremes of this value (Finch and Fairbairn 1991).

The reactor was continuously sampled by means of a peristaltic pump (flow rate - 9 mL/min). The sample was carried through a short piece of small-diameter Teflon tubing to a 35 µL flow cell with a light path of 1 cm that was located in a diode array spectrophotometer (Hewlett Packard model 8452A) set at a wavelength of 260 nm with a molar absorption coefficient of 3,300 M^{-1} cm^{-1}. The concentration factor was 14.55 cm • mg/L. After an adequate initial baseline had been obtained on the output graph, ozone was added to the reactor vessel. At the end of the desired contact time, 1.0 M sodium formate was used to neutralize the residual ozone. Sodium formate does not interfere with UV A_{260} measurements, and the reaction rate between sodium formate and ozone is very fast (Finch and Fairbairn 1991). Sampling of the reactor was continued to obtain a post-reaction baseline on the graph.

To calculate the applied ozone dose, three determinations of the aqueous ozone concentration in the stock solution were made before and two more after addition of ozone to the reactor vessel. An average of the five determinations was reported as the applied ozone concentration. Stock ozone concentrations were typically 18 to 24 mg/L. Appropriate amounts of ozone stock solution were added to the reactor vessels to achieve the desired applied dose. The applied ozone dose is the mass of the ozone obtained from the stock solution divided by the final volume of the reactor contents.

The initial ozone residual is the dissolved ozone observed in the reactor at time zero. The instantaneous ozone demand can be estimated from the ratio of the applied ozone dose to the initial ozone residual. The amount of ozone utilized during the contact period can be obtained by subtracting the final ozone residual from the applied ozone dose (Finch et al. 1993b).

Output graphs from the Hewlett Packard spectrophotometer were used to calculate the observed initial and final ozone residuals for each trial by multiplying the initial and final absorption values by the concentration factor (14.55 cm • mg/L). The observed initial ozone residual was typically 90-99% of the applied ozone dose. The final ozone residual was typically 83-90% of the inital ozone residual indicating that the amount of ozone decay was small over the short contact times used in this study.

For cold temperature experiments (3 °C), the reactor vessel was placed in a large beaker and surrounded with slush ice. A separate peristaltic pump was used to circulate water from a separate slush ice container through a cold temperature 35 µL flow cell to ensure that the flow cell was operating at the same temperature as the reactor vessel. Other than these modifications, the setup with regard to sampling the

reactor vessel to determine the ozone concentration with the Hewlett Packard spectrophotometer was identical to that just described above.

3.2.4. REACTOR VESSELS

Reactor vessels consisted of ODF 250 mL Erlynmeyer flasks with a Teflon coated magnetic stir bar for agitation. To zero the Hewlett Packard spectrophotometer, approximately 30 mL of ODF Milli Q water was poured into an ODF 50 mL Erlynmeyer flask. A peristaltic pump was used to pump the water through a flow cell. The spectrophotometer scanned the water sample to lock in a zero reading at 260 nm.

3.2.5. NONOZONATED CONTROL SAMPLES

After the appropriate amount of *L. innocua* stock solution had been added to the ODF phosphate buffer in the reactor vessel to give the desired initial bacterial concentration, the vessel was placed on a magnetic stirrer and thoroughly mixed. Three 9.9-10.1 mL control samples were removed and put in three dilution bottles containing 89-91 mL of sterile 1% peptone solution. Appropriate dilutions were carried out. The remaining 219.9-220.1 mL in the reactor vessel was then exposed to ozone.

3.2.6. OZONATED SAMPLES

At the end of the designed contact time, the residual ozone was quenched with 1 M sodium formate. Then either 3 or 4 samples were removed from the reactor and put in dilution bottles in a manner identical to that described for the control samples. For the cold temperature experiments, the temperature of the reactor contents was measured immediately after the last sample had been removed.

3.2.7. MEMBRANE FILTRATION AND PLATE COUNTS

L. innocua were enumerated by membrane filtration for both pre- and post-ozonation replicates using 0.45 μm MCE Black Filters (Gelman Sciences). *Standard Methods* membrane filter method (Method 9215) for heterotrophic plate counts was followed (APHA et al. 1992). *L. innocua* colonies were small but readily visible with a microscope after 48 hours of incubation on nutrient agar. Colonies had a pale blue color and showed up clearly against the black filter. Because plate counts were not significantly different ($P < 0.1$) for counts obtained after 48 and 72 hours of incubation, plates were counted after 48 hours of incubation at 20 °C. Experimental

data points were only considered valid if all the control and ozonated plate counts were between 20 and 200 colonies per plate.

3.2.8. VARIATION IN PLATE COUNTS

When replicates of a single sample are collected, it is assumed that the Poisson distribution describes the data adequately. To test the adequacy of the plating method, the distribution of data within a set of replicates was compared with the Poisson distribution using the D^2 statistic of Fisher. It is given by the following:

$$D^2 = \frac{(n-1)s^2}{\hat{x}} \qquad (1)$$

where s is the standard deviation of the replicates, n is the number of replicates and $\hat{x}$ is the mean of the replicates (Haas and Heller 1986). If the data is described by the Poisson distribution, the D^2 statistic is distributed as the χ^2 statistic with $n-1$ degrees of freedom. If the D^2 value is in excess of the tabulated χ^2 value for $n-1$ degrees of freedom at the selected confidence limit, then the initial assumption that the replicates can be described by Poisson distribution must be rejected (Haas and Heller 1986).

Three identical samples were collected prior to ozonation of *L. innocua*, and 3 or 4 samples were taken after ozonation. The D^2 statistic was applied to each set of pre- and post-ozonation replicates. The sets of replicates from experiments conducted at 22 °C had D^2 values less than the χ^2 tabulated value at the 10% level. The sets of replicates from experiments at 3 °C had D^2 values less than the χ^2 tabulated values at the 5% level. (See Appendix A for a complete listing of data and results from statistical analysis).

3.2.9. CONFIRMATION OF *L. INNOCUA* AFTER EXPERIMENTS

Upon completion of experiments, two diagnostic checks were carried out to verify that a foreign organism had not replaced *L. innocua* during propagation in TSBYE. A loopful of TSBYE containing *L. innocua* was spread on selective PALCAM agar. After 24 hours of incubation at 37 °C, the bacteria had produced a black color on the agar which is characteristic of *Listeria*. The black color is produced by the hydrolysis of esculin by *Listeria* (van Netten et al. 1989).

A motility test was also carried out. A test tube containing Tryptose agar (Difco) was stabbed and incubated at 20 °C. Growth occurred along the stab line and spread 3-5 mM below the surface of the agar in the shape of an open umbrella. This is characteristic of *Listeria* (Lovett 1989).

3.2.10. ESTIMATION OF MODEL PARAMETERS AND CONFIDENCE CONTOURS

The method of nonlinear least-squares was used to estimate the kinetic parameters of the model describing the inactivation data. The least-squares approach estimates the parameters by minimizing the least-squares residual (S):

$$S = \sum_{i=1}^{n} (y_i - u_i)^2 \tag{2}$$

where y_i is the observed survival ratio for each trial, u_i is the predicted survival ratio for each trial and n is the number of data points (Finch et al. 1993b). The solver routine of Microsoft Excel 4.0 (Microsoft Corp.) was used to minimize S for n data points that were given equal weights.

Joint parameter confidence regions can be estimated using the following equation (Draper and Smith 1981):

$$C = S\left\{1 + \frac{p}{n-p} F(p, n, 1-\alpha)\right\} \tag{3}$$

where S is the least squares residual determined by equation 3; n is the number of data points, p is the number of parameters, $F(p,n\text{-}p)$ is the F distribution with p and $n\text{-}p$ degrees of freedom . Although an exact confidence contour is defined by taking C as constant, because we do not know the correct distribution properties for a nonlinear model the probability level associated with the contour will have some uncertainty associated with it (Draper and Smith 1981). For a linear model, the contour would give an ellipsoidal shape, but nonlinear models may deviate from this.

4. Results and Discussion

In several recent ozone disinfection studies, the Hom model has been used to describe the inactivation results (Finch et al. 1993b; Haas et al. 1995). Because the Hom model appears to be the model of choice, it will be given priority in the analysis of the inactivation results from this study.

4.1. Description of Hom Model and Design Considerations

According to Chick's Law (Chick 1908) given as:

$$\frac{dN}{dt} = -kN \tag{4}$$

(where dN/dt is the inactivation rate, and N is the number of viable organisms), the plot of inactivation results should be linear. However, in practice two types of nonlinear inactivation curves have been encountered (Haas et al. 1995). "Shoulders" occur when there is a time lag before the onset of disinfection. The slope of the curve is gentle to begin with and then increases with the onset of inactivation, resulting in a curve that is concave downward. "Tailing off" occurs when the rate of inactivation is rapid to begin with but progressively decreases (ie. the curve drops sharply to begin with and then levels out like a curved "L"). When the m parameter in the Hom model is equal to 1, the equation reduces to the Chick-Watson model. For $m > 1$ shoulders are observed, and for $m < 1$, tailing-off behavior results (Haas et al. 1995).

Because deviations from the Chick-Watson model have been encountered, Hom developed an alternative model (Hom 1972). He began with a differential equation of the form:

$$\frac{dN}{dt} = -kNt^{m}C^{n} \tag{5}$$

Substituting $C^n = k'/t$ (where C is the concentration of disinfectant[1], n is the coefficient of dilution; k' represents the organism removal constant (mg*hrs/L); t = contact time (min.)) into the above equation and integrating yielded the following model:

[1]The initial observed ozone residual was typically 90-99% of the applied ozone dose and the final residual was typically 83-90% of the initial ozone residual. Since the decay of ozone was small over the short contact times used to inactivate *L. innocua*, the average ozone concentration (C) was determined by the method used in the study by Finch et.al, (1993a).

$$\log\frac{N}{N_o} = -\frac{Kk't^m}{m} \tag{6}$$

where N_o is the initial concentration of bacteria; N is the concentration of surviving bacteria; K is the first-order reaction rate constant per unit time to base ten (min.$^{-1}$); "m" is the m-order and n-order reaction rate constant. However, since $C^n = k't$, it can be substituted back into the above model to obtain:

$$\log\frac{N}{N_o} = -\frac{KC^n t^{m+1}}{m} \tag{7}$$

By defining $k = K/m$ and $m = m+1$ the following model is obtained:

$$\log\frac{N}{N_o} = -kC^n t^m \tag{8}$$

The literature in which the Hom model is discussed has not yet addressed experimental designs that will yield the most precise parameter estimates for k, m, and n. For example, it has been shown for the BOD model that concentrating the collection of data points at a point near $1/k$ and at a point well out on the curve near "infinity" will give much better parameter estimates than determining the BOD values over a large number of time intervals (Berthouex and Hunter 1971a; Berthouex and Hunter 1971b). Like the BOD model, the Hom model is inherently nonlinear, but significant differences arise between them due to the Hom model having three parameters.

In working with the data from this study, the parameter estimates for the Hom model using data points at $1/k$ and at "infinity" converged to extremely low or high values for k depending on the data set. When three data points were used, the model was ill-conditioned in that many local minima were obtained from the solver routine depending on the initial guesses for the parameters. Again, the parameter estimate for k tended to be very high. For the cold temperature model, data was collected at four points. When the data (including replicates) from all four points were used, the model stabilized and converged to a single minimum regardless of the initial guess values. This suggests that data should be obtained from at least four locations to avoid ill conditioning of the Hom model. A characteristic of nonlinear models is that some data points will influence the parameter estimates much more strongly than other data points. More work needs to be done in determining an optimum experimental design that will yield the most precise parameter estimates for the Hom model.

4.2. Experimental Results

The experiment conditions at 22 °C (pH 6.9) and the corresponding inactivation results are presented in Table 15. Experiments #16 and #18 were replicates using a low ozone dose and short contact time. Experiments #19 and #34 were replicates of a medium ozone dose and moderate contact time, while experiments #23 and #33 were replicates using a high ozone dose and longer contact time. A more detailed listing of the raw data is given in Appendix A.

Table 15 - Experimental Conditions and Inactivation of *L. innocua* in 0.1 M Phosphate Buffer at 22 °C

Experiment. Number	Contact Time (sec)	Applied Ozone Dose (mg/L)	Initial O_3 Residual (mg/L)	Initial Residual / Applied Dose Ratio	Final Ozone Residual (mg/L)	Average Ozone Dose (mg/L)	Survival Ratio (Log *N/No*)
7	10	0.0849	0.0737	0.87	0.0662	0.0698	-3.24
10	15	0.0421	0.0403	0.96	0.0281	0.0336	-3.20
11	10	0.0393	0.0384	0.98	0.0325	0.0353	-2.95
13	10	0.0188	0.0188	1.00*	0.0188	0.0188	-2.18
14	13	0.0103	0.0103	1.00*	0.0103	0.0103	-2.20
15	15	0.0479	0.0473	0.99	0.0403	0.0437	-3.60
16	10	0.0073	0.0073	1.00*	0.0073	0.0073	-1.97
18	10	0.0077	0.0077	1.00*	0.0077	0.0077	-2.15
19	30	0.0454	0.0409	0.90	0.0284	0.0341	-3.54
23	63	0.1232	0.1112	0.90	0.0921	0.1012	-4.70
33	64	0.1245	0.1220	0.98	0.1019	0.1115	-4.83
34	30	0.0456	0.0418	0.92	0.0354	0.0385	-3.84

* Ozone dose is below the detection limits of the spectrophotometer, so the initial and final residuals were assumed to be equal to the applied dose which is reasonable with contact time of only 10 seconds.

The conditions for experiments conducted at 3 °C are presented along with the inactivation results in Table 16. Replicates were obtained at low, medium and high ozone doses to allow the calculation of pure experimental error. A complete listing of the raw data is included in Appendix A.

Table 16 - Experimental Conditions and Inactivation of *L. innocua* in 0.1 M Phosphate Buffer at 3 °C

Experiment Number	Contact Time (sec)	Applied Ozone Dose (mg/L)	Initial O_3 Residual (mg/L)	Initial Residual / Applied Dose Ratio	Final Ozone Residual (mg/L)	Average Ozone Dose (mg/L)	Survival Ratio (Log N/N_o)
27	30	0.0457	0.044	0.97	0.0292	0.0360	-3.59
36	30	0.0457	0.0419	0.92	0.0377	0.0397	-3.12
40	30	0.0454	0.0444	0.98	0.0382	0.0412	-3.80
49	63	0.1058	0.1048	0.99	0.0889	0.0965	-4.58
50	10	0.0075	0.0075	1.00*	0.0075	0.0075	-1.93
52	10	0.0075	0.0075	1.00*	0.0075	0.0075	-1.84
53	63	0.1040	0.1020	0.98	0.0864	0.0939	-5.15
55	63	0.1032	0.1012	0.98	0.0867	0.0937	-4.39
64	46	0.0749	0.0736	0.98	0.0585	0.0656	-3.88

* Ozone dose is below the detection limits of the spectrophotometer, so the initial and final residuals were assumed to be equal to the applied dose which is reasonable with only a 10 second contact time.

4.3. Parameter Estimates for the Hom Model

4.3.1. PARAMETER ESTIMATES FOR 22 °C DATA

The parameter estimates obtained for the Hom model parameters k, n, and m were determined for the data in Table 15 using nonlinear least squares. The resulting equation was:

$$\log\frac{N}{N_o} = -7.79C^{0.21}T^{0.15} \tag{9}$$

To gain an idea of the response surface of *L. innocua* to ozone disinfection predicted by the Hom model, equation 10 was used to generate a table of survival ratios ($\log(N/N_o)$) based on a range of selected values for the average ozone dose (C) and contact time (T). A plot of the response surface is depicted in Figure 2.

The Hom model parameter estimates from this study are compared with parameter estimates for other microorganisms - see Table 17. *L. innocua* has the highest value for the reaction rate constant, k, suggesting that it is the least resistant organism to ozone disinfection. The parameter estimates for *L. innocua* are the most similar to those obtained for *E. coli*. *E. coli* is recognized as being quite sensitive to

inactivation by ozone (Bablon et al. 1991b), and it appears that *L. innocua* is even less resistant to ozone disinfection.

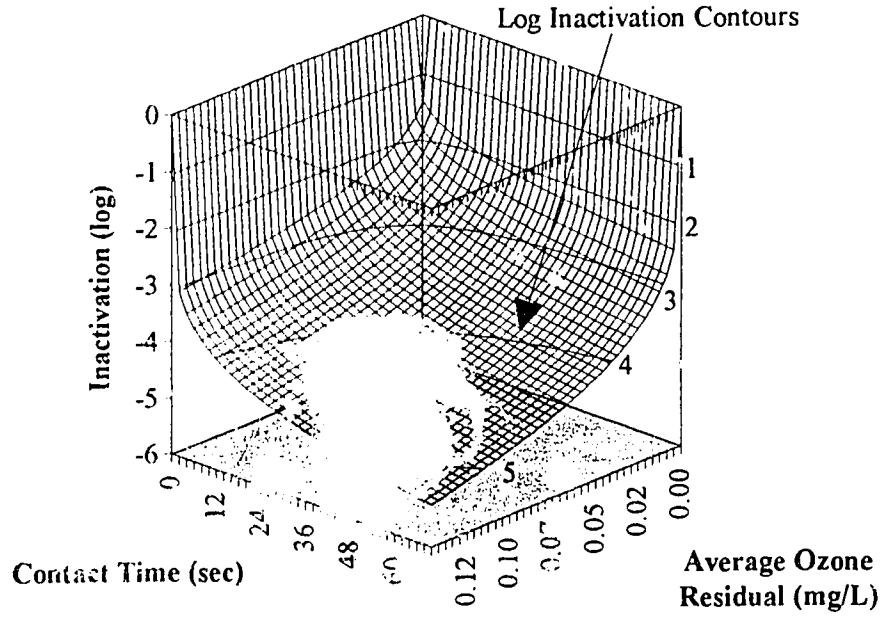

Figure 2 - Response Surface of *L. innocua* Inactivation Predicted by the Hom Model as a Function of Ozone Residual and Contact Time in Phosphate Buffer at 22 °C

The Hom model parameter estimates for *E. coli* shown in Table 17 were determined from data in a previously published paper (Finch and Smith 1990). The initial concentration of *E. coli* was 1.7×10^5 CFU/mL. This concentration compares favorably with the initial concentrations used in this study which ranged from 5.53×10^5 to 1.34×10^6 CFU/mL. More than 5 logs of *E. coli* inactivation were observed when an ozone dose of 0.2 mg/L was applied for a contact time of 60 seconds (Finch and Smith 1990). In Table 15, just under 5 logs of *L. innocua* inactivation was obtained with an average ozone dose of 0.1 mg/L and a contact time of 63 seconds. Comparison of these data points from both studies suggests that a 5 log inactivation of *L. innocua* requires approximately half as much ozone as that required for a similar inactivation of *E. coli*.

Table 17 - Comparison of Hom Model Parameter Estimates

Organism	Hom Mod. Parameters			N_0	Temp.	Source
	k	*n*	*m*	(Org. / mL)	(°C)	
L. innocua	7.79	0.21	0.15	$5x10^5$ to $1x10^6$	22	This study
HPC bacteria	2.19	0.22	0.20	$\approx 1x10^4$	22	(Gyurek 1995)
E. coli	6.67	0.13	0.14	$\approx 2x10^5$	20	(Gyurek 1995)
Giardia lamblia and *G. muris*	1.04	-0.84	0.12	$1x10^6$	22	(Finch et al. 1993b)
Cryptosporidium parvum	0.82	0.23	0.64	$8x10^3$ to $2x10^4$	22	(Finch et al. 1993a)

Two tests can be applied to gain information about the adequacy of a nonlinear model to describe data. First, the model can be examined to see if the lack of fit between model predictions and data points are statistically significant. Secondly, the residual plots can be examined to see if they exhibit constant variance.

Table 18 presents the analysis of variance results obtained for the Hom model for the 22 °C data. The residual error of the model was partitioned into lack-of-fit and pure error. The pure error was determined from replicates of low, medium and high ozone doses. Statistically the model is shown to be adequate because the lack-of-fit to pure error ratio is not significant at the 5% level.

Table 18 - ANOVA of the Hom Model for Inactivation of *L. innocua* at 22 °C

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Model	3	132.691	44.23	821.6*
Error	9	0.485	0.054	
Total	12	133.176		
Lack-of-fit	6	0.418	0.0697	3.14**
Pure error	3	0.067	0.0222	
Total	9	0.485		

* Significant at 0.1% level

** Not significant at 5% level

The second check of a model's adequacy is based on examination of the residual error. Residual plots from a fully adequate model should exhibit constant variance. If the residuals exhibit a curved pattern, it may indicate that the model is missing a parameter or that a transformation of the data should be carried out before analysis (Draper and Smith 1981). Residuals should be plotted against the independent variables and the results predicted by the model (Draper and Smith 1981).

The residuals from the Hom Model were plotted against the predicted survival ratio (Figure 3), time (Figure 4) and ozone dose (Figure 5). Taken by itself, the curved pattern in the plots would raise questions about the adequacy of the model. However, the parameter estimates from the warm and cold temperature experiments were very similar (See Tables 17 and 19). When the two data sets are plotted on the same graph (Figure 7), the results are very similar. The residual error plots from the cold temperature (Figures 9, 10, and 11) data do not appear to exhibit a marked curvature like the warm temperature (22 °C) plots. Because parameter estimates obtained from nonlinear least squares can be heavily influenced by a single data point, it appears that this type of phenomenon may have occurred in the case of the data obtained at 22 °C.

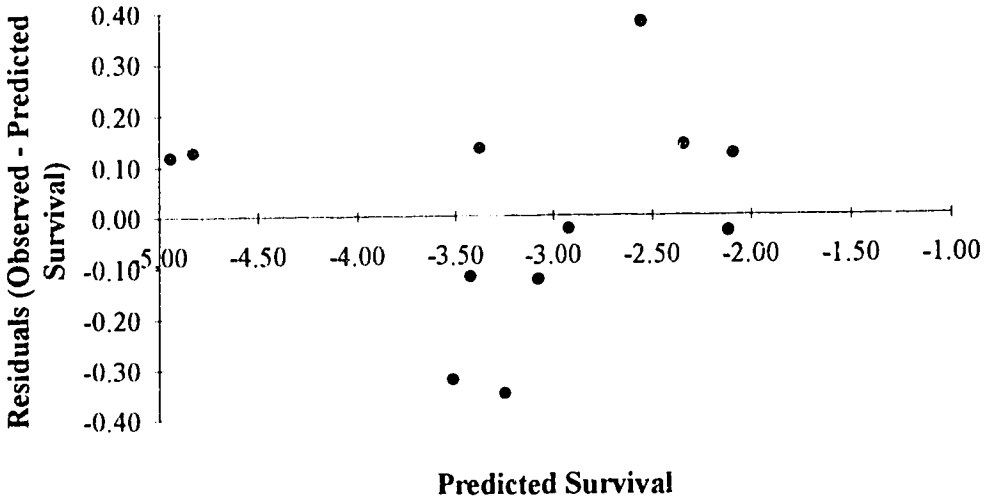

Figure 3 - Residuals Plot from Hom Model for Inactivation of *L. innocua* at 22 °C (Residuals vs. Predicted Survival)

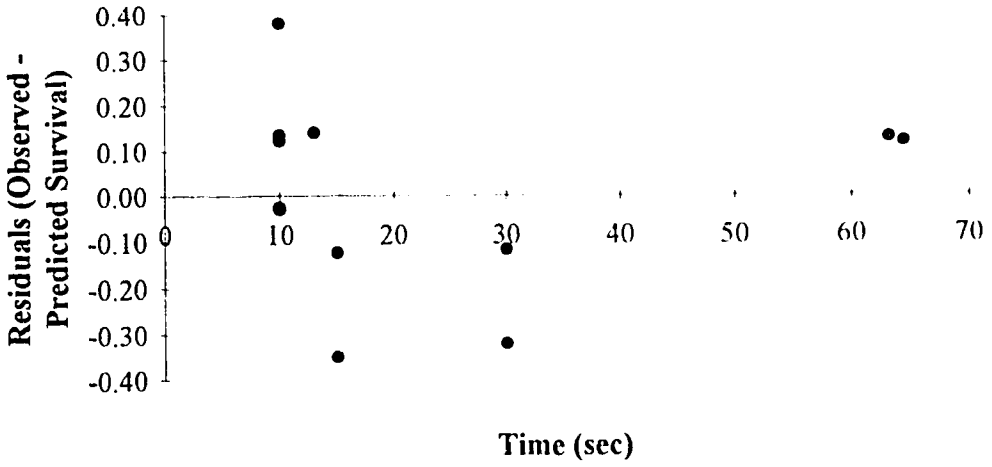

Figure 4 - Residuals Plot from Hom Model for Inactivation of *L. innocua* at 22 °C (Residuals vs. Time)

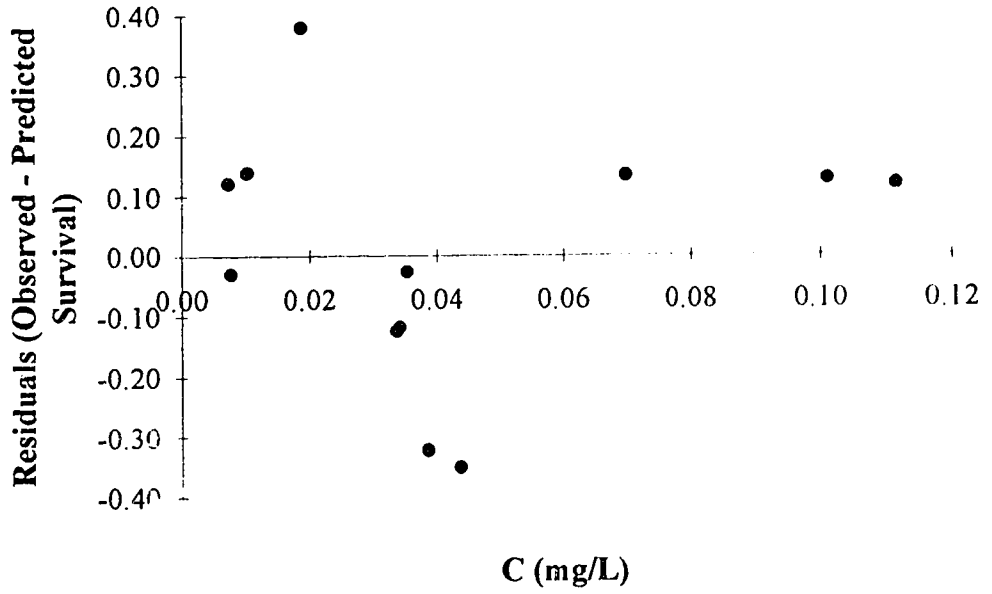

Figure 5 - Residual Plot from Hom Model for Inactivation of *L. innocua* at 22 °C (Residuals vs. Ozone Concentration)

One of the goals in fitting a model to a set of experimental data is to have parameter estimates that are precise. An idea of the precision of the parameter estimates can be obtained from examination of the joint confidence region for the

parameters. A model with two parameters will have a two-dimensional joint confidence region. The joint confidence region for a model with three parameters consists of a volume. A joint confidence region is constructed by computing nonlinear least sums of squares values for the data set over an appropriate region by varying each of the parameter estimates. Equation 4 is used to compute the value at which the contour is evaluated that is associated with an approximate probability level for nonlinear models.

A three dimensional confidence region is difficult to construct. Instead, approximate confidence limits for the parameters can be obtained by generating two-dimensional slices of the volume using nonlinear least squares. Such a slice is presented in Figure 6, where the maximum variation for the *k* and *n* parameters is depicted.

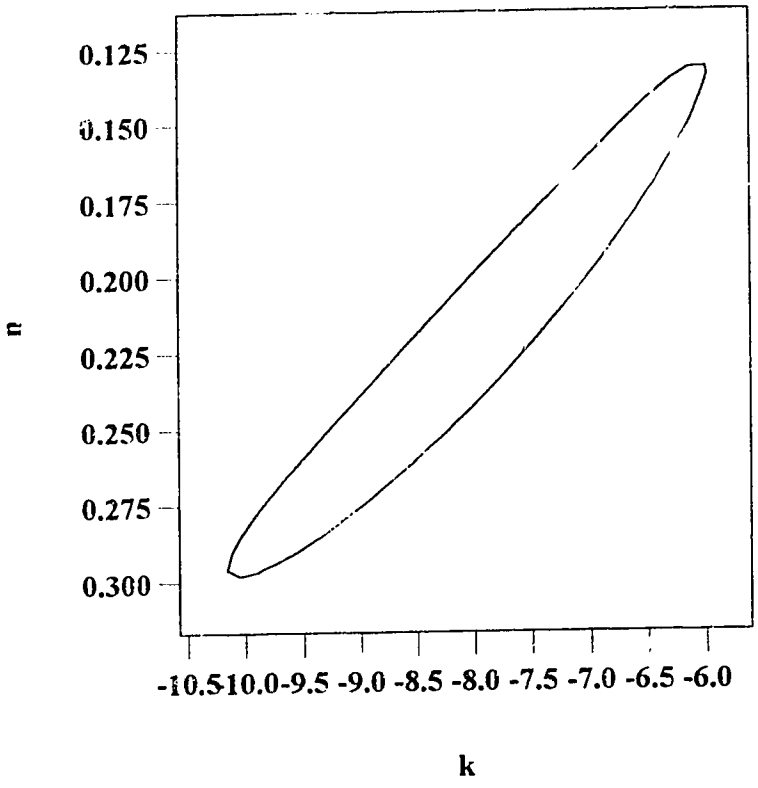

Figure 6 - Slice of 95% Confidence Region for 22 °C Hom Model Parameters Depicting Maximum Variation for Parameters *k* and *n* while holding *m* Constant at its Minimum Value

The slice was generated by varying the k and n parameters and holding the m parameter at its minimum value. The confidence interval for the m parameter was obtained by generating slices of the confidence region by incrementing the value of m and continuing to vary the values of k and n. These additional slices demonstrated that the shape of the joint confidence region was ellipsoidal in nature. From Figure 6 and the other slices, the following 95% confidence limits were obtained for the Hom model parameters for the data obtained at 22 °C:

$$-6.0 < k < -10.1$$

$$0.13 < n < 0.30$$

$$0.03 < m < 0.29$$

One way of comparing the warm and cold temperature data sets is by plotting the survival ratio against the product of the average ozone residual and contact time (Figure 7). Both data sets exhibit the tailing effect often seen with viral and bacteria inactivation curves (Finch et al. 1988; Katzenelson et al. 1974; Roy et al. 1981; Roy et al. 1982).

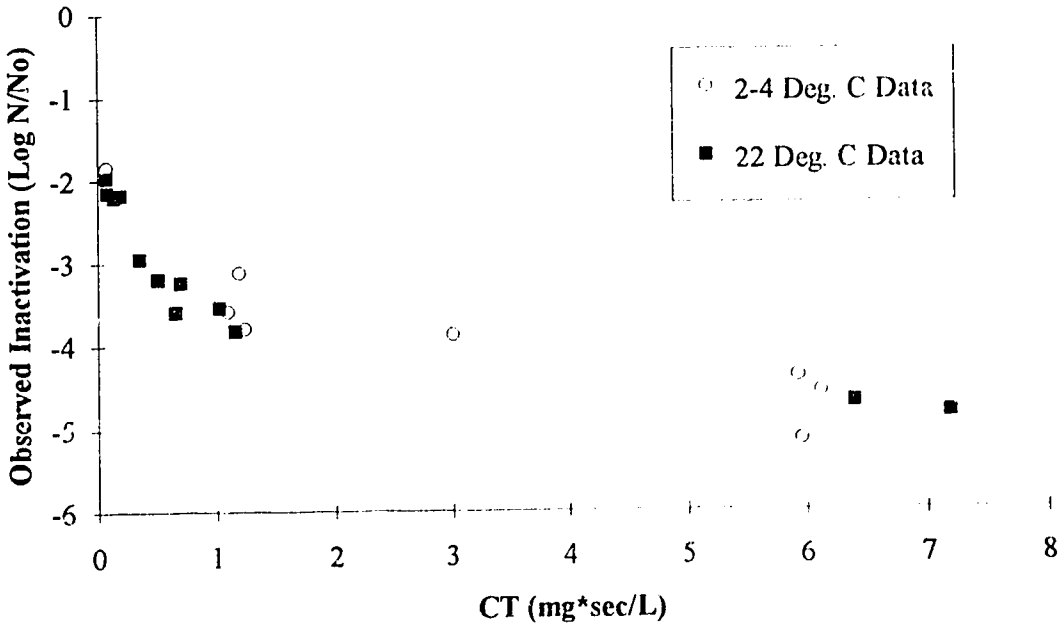

Figure 7 - Comparison of *L. innocua* Inactivation at 22 °C and 3 °C as a Function of the Average Ozone Residual x Contact Time Product

The results depicted in Figure 7 also indicate that there was little difference between the resistance of *L. innocua* to inactivation by ozone at 3 °C and 22 °C. Unlike *N. gruberi*, *G. muris*, and *G. lamblia* cysts and six enteroviruses that were more sensitive to ozone at lower temperatures (Roy et al. 1982; Wickramanayake et al. 1984a; Wickramanayake et al. 1984b), and unlike *M. fortuitum*, *C. parvum*, hepatitis A virus, poliovirus 1 and *E. coli* that were less resistant to ozone at higher temperatures (Farooq et al. 1977b; Finch et al. 1993a; Herbold et al. 1989), *L. innocua* was similar to poliovirus 1, MS2 bacteriophage, HPC bacteria in that they demonstrated a similar resistance to ozone inactivation at warm and cold temperatures (Helmer and Finch 1993; Herbold et al. 1989).

4.3.2. PARAMETER ESTIMATES FOR 3 °C DATA

Hom model parameter estimates for k, n, and m were determined for the data in Table 16 using nonlinear least squares. The resulting equation was:

$$\log\frac{N}{N_o} = -8.02C^{0.23}T^{0.16} \qquad (19)$$

Similar to what was done for the warm temperature model, Equation 11 was used to generate a table of survival ratios [$\log(N/N_o)$] based on a range of selected values for the average ozone dose (C) and contact time (T). A plot of the response surface is depicted in Figure 8. The response surface depicted in Figure 8 is very similar to the response surface obtained for the warm temperature data in Figure 2.

The Hom model parameter estimates from this study are compared with parameter estimates for *C. parvum* - see Table 19. The reaction rate constant (k) is much higher for *L. innocua* than for *C. parvum* which indicates that *L. innocua* is much less resistant to ozone disinfection than *C. parvum*. This is seen even more clearly when points along the inactivation curves are compared. An average ozone dose of 0.09 mg/L with a contact time of 63 seconds resulted in a 5.1 log inactivation of *L. innocua*. In contrast to this an average ozone dose of 0.9 mg/L (10 times higher) with a contact time of 15 minutes resulted in a 3.6 log inactivation of *C. parvum* at 8 °C. The product of ozone dose and contact time is over 140 times greater for *C. parvum* than for *L. innocua*. This clearly demonstrates the much greater sensitivity of *L. innocua* to ozone than that of *C. parvum*.

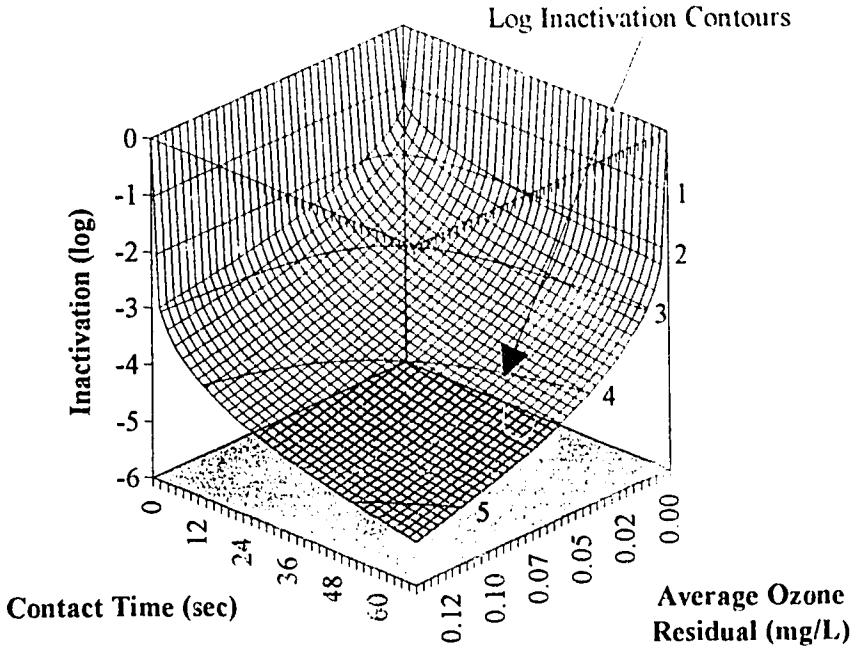

Figure 8 - Response Surface of *L. innocua* Inactivation Predicted by the Hom Model as a Function of Ozone Residual and Contact Time in Phosphate Buffer at 3 °C

Table 19 - Hom Model Parameter Estimates for Experiments at Cold Temperatures

Organism	Hom Mod. Parameters			N_o	Temp.	Source
	k	*n*	*m*	(Org. / mL)	(°C)	
L. innocua	8.02	0.23	0.16	$2x10^5$ to $2x10^6$	3	This study
C. parvum	0.29	0.68	0.95	$3x10^3$ to $2x10^4$	7	(Finch et al. 1993a)

To check the adequacy of the Hom model to describe the cold temperature data, the model was tested for lack-of-fit. Table 20 presents the analysis of variance results obtained from the model for the 3 °C data. The model residual error was partitioned into lack-of-fit and pure error. The pure error was determined from replicates of low, medium and high ozone doses. The lack-of-fit does not raise

questions about the adequacy of the model because the lack-of-fit to pure error ratio is not significant at the 10% level.

Table 20 - ANOVA of the Hom Model for Inactivation of *L. innocua* at 3 °C

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Model	3	125.420	41.806	388.8*
Error	6	0.645	0.1075	
Total	9	126.065		
Lack-of-fit	1	0.088	0.0880	0.79**
Pure error	5	0.557	0.1114	
Total	6	0.645		

* Significant at 0.1% level
** Not significant at 10% level

The residual error from the Hom Model for the 3 °C data was plotted against the predicted survival ratio (Figure 9), time (Figure 10) and ozone dose (Figure 11). The residuals do not have the curved pattern that characterized the residual plots from the data obtained at 22 °C.

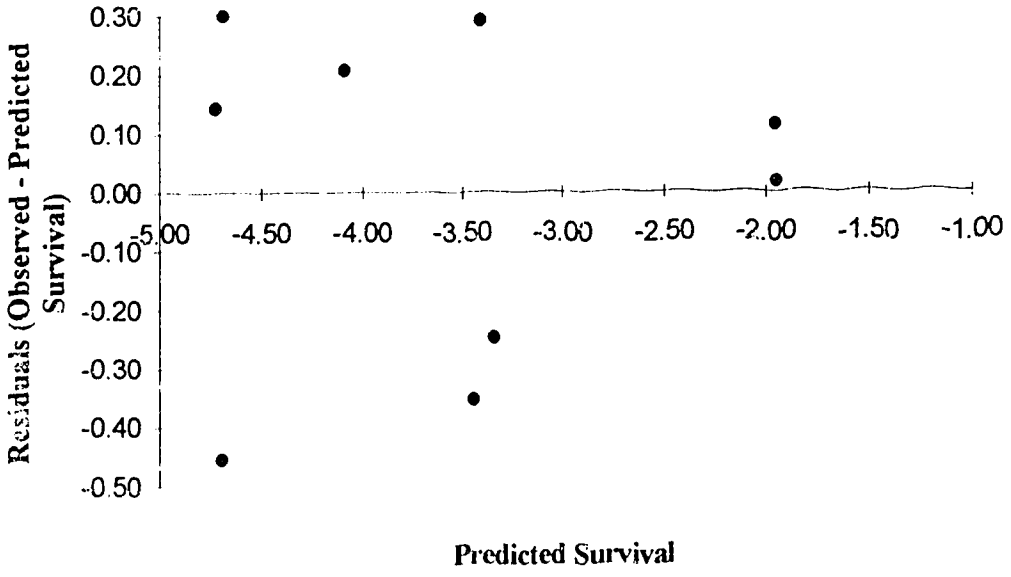

Figure 9 - Residuals Plot from Hom Model for Inactivation of *L. innocua* at 3 °C (Residuals vs. Predicted Survival)

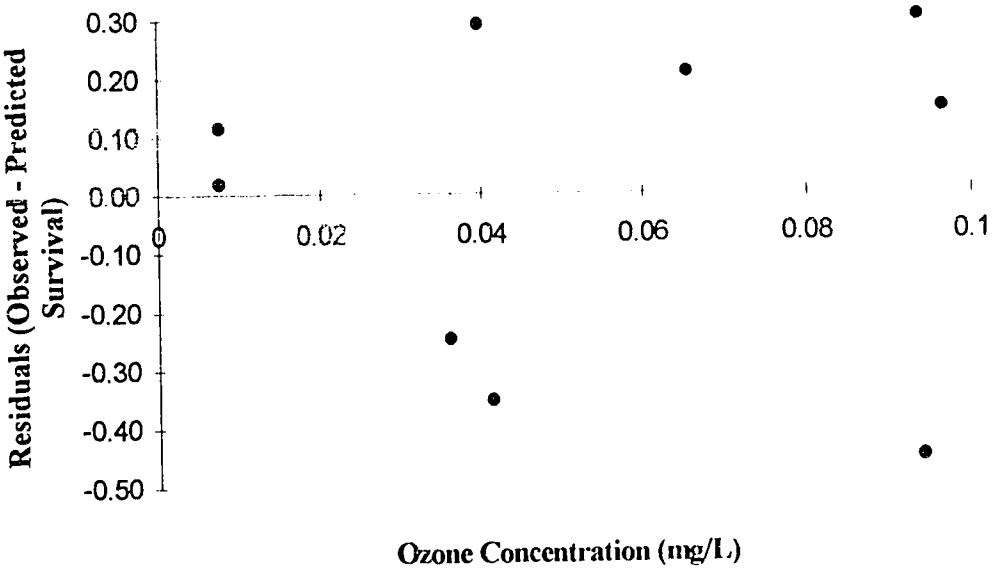

Figure 10 - Residuals Plot from Hom Model for Inactivation of *L. innocua* at 3 °C (Residuals vs. Ozone Concentration)

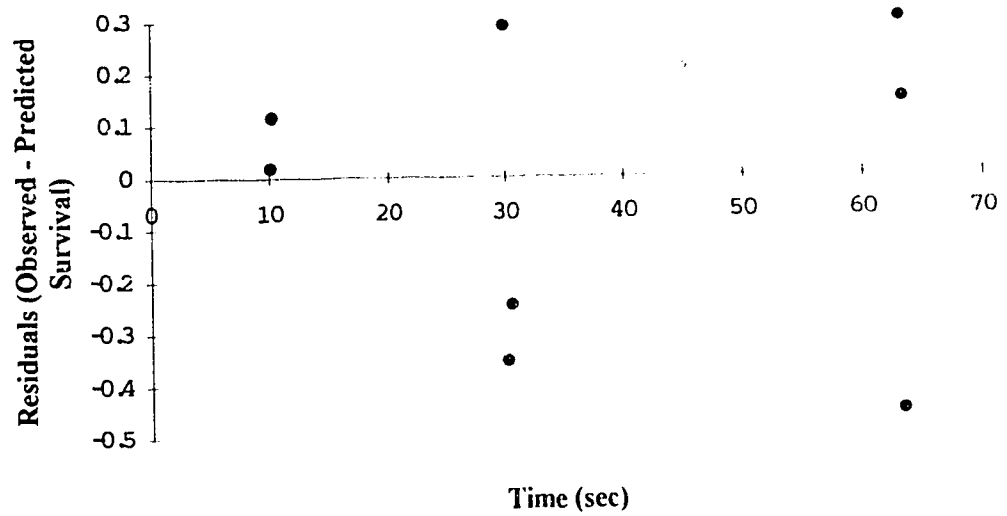

Figure 11 - Residuals Plot from Hom Model for Inactivation of *L. innocua* at 3 °C (Residuals vs. Time)

The joint confidence region for the cold temperature parameter estimates was computed in a similar fashion to that described for the warm temperature data. A two dimensional slice of the 95% joint confidence region was obtained by holding m constant and varying k and n is shown in Figure 12. However, the figure is misleading in that the actual volume is a large crescent moon shaped object. Examination of additional cross sectional slices where m and k were varied and n was incremented reveals that the 95% confidence limits for the parameters are greater than the following:

$$-29.6 < k < -2.2$$
$$-0.20 < n < 0.80$$
$$-0.49 < m < 0.87$$

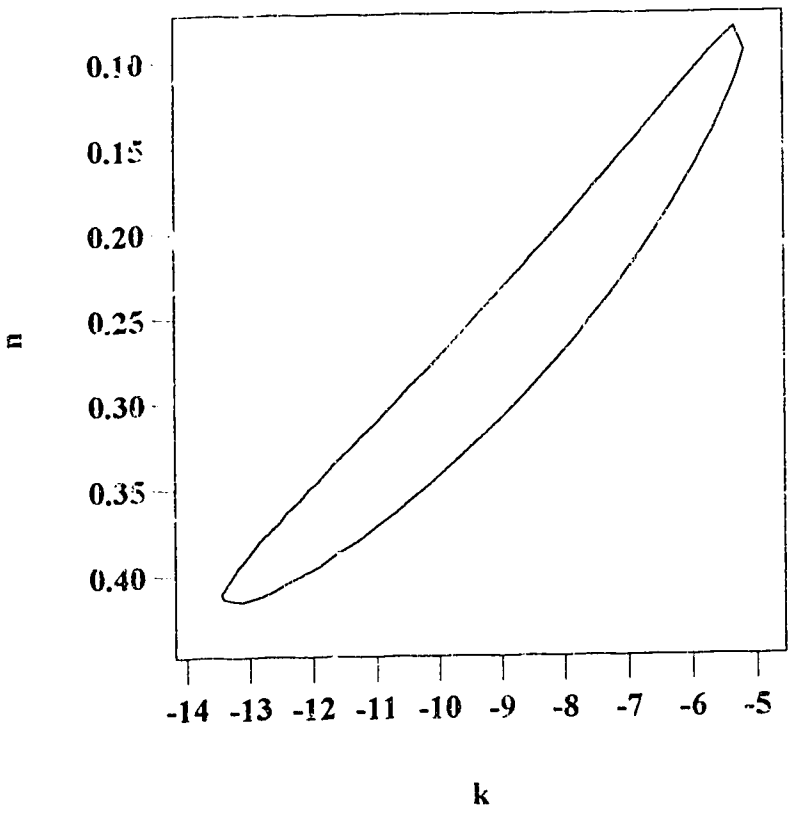

Figure 12 - Slice of 95% Confidence Region for 3 °C Hom Model Parameters Depicting Variation for Parameters k and n While Holding m Constant at its Minimum Value

The large joint confidence region suggests that the data set may have some ill conditioning. This could have occurred for several reasons. First, even though there

were 9 data points, because replicates were performed at low, medium and high ozone doses to allow for the estimation of the pure error in the experiments, this had the effect of reducing the number of points in the experimental region at which data was collected.

A second potential cause of the large joint confidence region could arise from Equation 5 which was used to generate the confidence contour. This equation is somewhat sensitive to the size of the p to n-p ratio. Larger p to n-p ratios occur when there are fewer data points and a larger number of parameters resulting in a larger value of C (the value at which the joint confidence contour is evaluated). The larger the value for C, the larger will be the size of the joint confidence region.

A third reason for the large confidence region comes from comparison of the size of the pure error term in Tables 18 and 20. The pure error for 3 °C data was 0.557, while the pure error for the 22 °C data was only 0.067. The pure error term stems directly from the amount of variance between data points within replicates. The greater the degree of variance within replicates, the greater will be the degree of imprecision in the parameter estimates.

4.3.3. EFFECT OF INITIAL CONCENTRATION OF BACTERIA ON INACTIVATION RESULTS

During initial work to establish experimental protocols, a higher initial concentration of *L. innocua* was used. Later it was observed that a 2 log inactivation in experiments with a higher initial concentration required a much greater ozonation effort than when the initial concentration was in the $5x10^5$ CFU/mL range. This led to conducting some experiments where the initial concentration of organisms ranged from 2.5×10^7 to 3.7×10^7 CFU/mL. The experimental conditions and inactivation results are presented in Table 21. Increasing the initial concentration of *L. innocua* to this level rules out the use of spectrophotometry to measure initial and final ozone residuals due to the fact that the high concentration of organisms interferes with UV absorbance. In fact, the baseline on the output graphs from the spectrophotometer was not equal to zero, and no peaks were observed on the graphs when ozone was added. Rather the curve decreased after the addition of ozone, and this was possibly due to the clearing of the solution as cells were lysed.

Table 21 - Experimental Conditions and Inactivation of *L. innocua* in 0.1 M Phosphate Buffer at 22 °C with $N_o \approx 2.9 \times 10^7$ CFU/mL

Experiment Number	Contact Time (sec)	Applied Ozone Dose (mg/L)	Initial Concentration (CFU/mL)	Survival Ratio (Log *N/No*)
56	30	0.0449	2.5×10^7	-1.83
57	63	0.1290	3.1×10^7	-3.28
58	180	0.4489	2.4×10^7	-4.96
60	120	0.2206	2.8×10^7	-4.09
62	63	0.1295	3.7×10^7	-3.67

Because initial and final ozone residuals were not measured for experiments with higher initial concentrations, definitive comparisons of results between experiments with lower and higher initial concentrations is not possible. However, since the same ozone dose and contact time were applied in some of the experiments, a qualitative comparison is possible. In Table 15, a 30 sec. contact time and an applied ozone dose of 0.041 mg/L resulted in a log survival ratio of -3.69 (Exp. #19 and #34). In Table 21, a similar ozone dose and contact time resulted in a log survival ratio of only -1.83. Likewise, in Table 15, a 63 sec. contact time and an applied ozone dose of approximately 0.117 mg/L resulted in a log survival ratio of -6.79. But in Table 21, similar experimental conditions only gave on average a log survival ratio of -3.47.

Another qualitative comparison of the inactivation results based on the applied ozone dose and contact time product is shown in Figure 13. The graph indicates that to achieve a 5 log kill with $N_o \approx 3 \times 10^7$ CFU/mL an applied ozone and contact time product approximately 8 times greater than the product for $N_o \approx 7 \times 10^5$ CFU/mL was required.

One explanation for the different levels of inactivation associated with different initial concentrations of *L. innocua* could involve the residual ozone dose over the contact period. Even though similiar ozone doses were *applied* in some of the experiments, the *residual* ozone dose was not measured in experiments with initial concentrations of 10^7 CFU/mL. A larger ozone dose quite likely was needed in experiments with higher initial concentrations to achieve the same average ozone dose used in the experiments with lower initial concentrations. In addition, if the triple

experiments with with $N_o \approx 10^7$ CFU/mL due to carry over of the culture media. The concentration of culture media carrried over from the washing process would have been 100 times greater when the intial concentration of organisms was 10^7 CFU/mL than when $N_o \approx 7 \times 10^5$ CFU/mL.

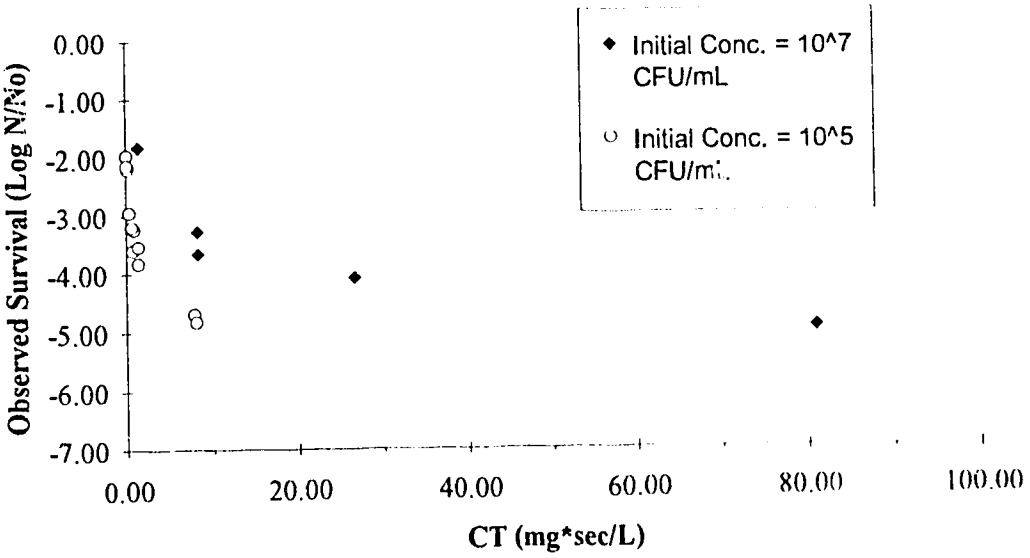

Figure 13 - Comparison of *L. innocua* Inactivation with Different Initial Bacterial Concentrations as a Function of the *Applied* Ozone Dose x Contact Time Product

Another facter that bears consideration is the fraction of bacteria that are more resistant to ozone. A certain portion of a bacteria population will be more resistant to disinfection. By increasing the initial concentration of *L. innocua* in the reactor vessel, the corresponding increase in the resistant fraction of *L. innocua* could have had some effect of the inactivation results.

While these explanations may account for the difference in levels of inactivation when the initial concentration of bacteria was increased, they might not account for all of the variance in the outcomes. It might be possible that the survival ratio term (N/N_o) found in most inactivation models may not be independent of the initial concentration of organisms. If this is the case, inactivation model parameters may only be valid over the experimental region in which they are established. Further work is needed to verify this observation since the work done here does not in any way address the depletion of ozone during the contact time for experiments with 10^7 CFU/mL.

Conclusions and Recommendations

4.4. Conclusions

The work performed for this project accomplished the three research objectives. Hom model parameters were estimated from the data collected at 3 °C and 22 °C by using the method of nonlinear least squares. Although the analysis of variance from the models indicated no significant lack-of-fit, some questions about the models' adequacy arose from curvature in the residual plots for the 22 °C data. The precision of the parameter estimates was acceptable for the 22 °C data, but a greater degree of precision for the cold temperature parameter estimates would have been desirable.

Ozone disinfection of *L. innocua* at 22 °C and at 3 °C indicated that a similar level of inactivation occurred in experiments conducted at both temperatures. Virtually identical Hom model parameter estimates were obtained at the two temperature settings. Compared with other microorganisms, *L. innocua* appears to be very sensitive to ozone.

Comparison of inactivation results when the initial concentration of organisms is increased 100 fold indicated that a much greater ozonation effort is required to achieve the same level of inactivation. Although this may be due to differences in residual ozone doses, it also might be possble that the initial concentration of organisms has an effect on the inactivation results. If this is so, parameter estimates from models employing a survival ratio term ($\log(N/N_o)$) may be limited to the experimental region in which they were determined.

4.5. Recommendations

The findings from this study indicate that *L. innocua* and by association *L. monocytogenes* are very sensitive to ozone disinfection. This suggests the possibility that some easily inactivated pathogenic microorganisms could be eliminated from poultry meat if ozone was used as a disinfectant during processing.

The response of a significant number of poultry pathogens to ozone disinfection is not known. Future research could focus on the pathogens that pose greater health risks such as *Campylobacter jejuni*. Knowledge of the relative

resistance of pathogenic organisms to ozone disinfection would allow the most resistant organism to be used as a criterion of disinfection adequacy.

Because past researchers have used a variety of ozone protocols and methods to report the results, a precise ranking of microorganisms' resistance to ozone disinfection is difficult to ascertain from the literature. Use of a consistent ozone protocol and analytical model such as the Hom model in future research would greatly facilitate the comparison of results between researchers.

Further research should be conducted to determine experimental design conditions that will give the best parameter estimates for the Hom model. Another area for further exploration is determining what effect (if any) the initial concentration of bacteria (N_o) has on the Hom model parameters and the region for which the parameter estimates are valid.

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Appendix A - Analytical Data

Experiment Number	DA022007	Experiment Number	DA022710	Experiment Number	DA022711
O3 conc. #1 (mg/L)	19.12	O3 conc. #1 (mg/L)	19.05	O3 conc. #1 (mg/L)	17.64
O3 conc. #2 (mg/L)	18.99	O3 conc. #2 (mg/L)	18.79	O3 conc. #2 (mg/L)	17.42
O3 conc. #3 (mg/L)	18.53	O3 conc. #3 (mg/L)	18.52	O3 conc. #3 (mg/L)	17.38
O3 conc. #4 (mg/L)	18.66	O3 conc. #4 (mg/L)	18.18	O3 conc. #4 (mg/L)	17.13
O3 conc. #5 (mg/L)	18.42	O3 conc. #5 (mg/L)	18.41	O3 conc. #5 (mg/L)	17.28
AVERAGE (mg/L)	18.74	AVERAGE (mg/L)	18.59	AVERAGE (mg/L)	17.37
Vol. of soln. (mL)	219.800	Vol. of soln. (mL)	220.4	Vol. of soln. (mL)	220.4
Vol. of stock O3 (mL)	1.000	Vol. of stock O3 (mL	0.500	Vol. of stock O3 (mL	0.500
Applied concen. (mg/L)	0.0849	Appl. conc. (mg/L)	0.0421	Appl. conc. (mg/L)	0.0393
Contact time (sec)	10	Contact time (sec)	15	Contact time (sec)	10
Peak on graph	-0.00327	Peak on graph	0.00891	Peak on graph	0.00967
Baseline on graph	-0.00833	Baseline on graph	0.00614	Baseline on graph	0.00703
Difference	0.00506	Difference	0.00277	Difference	0.00264
Observed concen. (mg/L	0.0737	Obs. conc. (mg/L)	0.0403	Obs. conc. (mg/L)	0.0384
Final peak on graph	-0.00378	Final peak on graph	0.00807	Final peak on graph	0.00926
Difference	0.0047	Difference	0.00193	Difference	0.00223
Residual concen. (mg/L)	0.0662	Resid. conc. (mg/L)	0.0281	Resid. conc. (mg/L)	0.0325
Integrated Conc. (mg/L)	0.069843	Integ. Conc. (mg/L)	0.0336	Integ. Conc. (mg/L)	0.0353
C observed / C applied	0	C obs. / C appl.	0.96	C obs. / C appl.	0.98
Control Plate counts:		Control Plate counts:		Control Plate counts:	
47C Plate #1	55	410C Plate #1	65	410C Plate #1	52
47C Plate #2	77	410C Plate #2	50	410C Plate #2	63
47C Plate #3	59	410C Plate #3	56	410C Plate #3	51
Average	63.7	Average	57.0	Average	55.3
Standard Deviation	11.72	Standard Deviation	7.55	Standard Deviation	6.66
Variance	137.33	Variance	57.00	Variance	44.33
D^2	4.31	D^2	2.00	D^2	1.60
Within 90% Chi2 dist.	Yes	w/in 90% Chi2 dist.	Yes	w/in 90% Chi2 dist.	Yes
Dilution factor	1.00E+04	Dilution factor	1.00E+04	Dilution factor	1.00E+04
Experiment Plate Counts:		Experiment Plate Counts:		Experiment Plate Counts:	
17E Plate #1	37	110E Plate #1	29	111E Plate #1	70
17E Plate #2	36	110E Plate #2	34	111E Plate #2	59
17E Plate #3	36	110E Plate #3	45	111E Plate #3	58
Average	36.3	Average	36.0	Average	62.3
Standard Deviation	0.58	Standard Deviation	8.19	Standard Deviation	6.66
Variance	0.33	Variance	67.00	Variance	44.33
D^2	0.02	D^2	3.72	D^2	1.42
Within 90% Chi2 dist.	Yes	Within 90% Chi2 di	Yes	Within 90% Chi2 di	Yes
Dilution factor	1.00E+01	Dilution factor	1.00E+01	Dilution factor	1.00E+01
Log Kill:		Log Kill:		Log Kill:	
No	6.37E+05	No	5.70E+05	No	5.53E+05
N	3.63E+02	N	3.60E+02	N	6.23E+02
Log N/No	-3.24	Log N/No	-3.20	Log N/No	-2.95
Valid Data Point	Yes	Valid Data Point	Yes	Valid Data Point	Yes
CT	0.698	CT	0.505	CT	0.353

Experiment Number	DA030613	Experiment Number	DA030814	Experiment Number	DA030815
O3 conc. #1 (mg/L)	16.93	O3 conc. #1 (mg/L)	14.58	O3 conc. #1 (mg/L)	21.76
O3 conc. #2 (mg/L)	16.59	O3 conc. #2 (mg/L)	14.41	O3 conc. #2 (mg/L)	21.32
O3 conc. #3 (mg/L)	16.67	O3 conc. #3 (mg/L)	14.48	O3 conc. #3 (mg/L)	20.98
O3 conc. #4 (mg/L)	16.37	O3 conc. #4 (mg/L)	14.25	O3 conc. #4 (mg/L)	20.96
O3 conc. #5 (mg/L)	16.37	O3 conc. #5 (mg/L)	14.09	O3 conc. #5 (mg/L)	20.73
AVERAGE (mg/L)	16.59	AVERAGE (mg/L)	14.36	AVERAGE (mg/L)	21.15
Vol. of soln. (mL)	220.4	Vol. of soln. (mL)	220.458	Vol. of soln. (mL)	220.4
Vol. of stock O3 (mL)	0.250	Vol. of stock O3 (mL	0.158	Vol. of stock O3 (mL	0.500
Applied concen. (mg/L)	0.0188	Appl. conc. (mg/L)	0.0103	Appl. conc. (mg/L)	0.0479
Contact time (sec)	10	Contact time (sec)	13	Contact time (sec)	15
Peak on graph	N/A	Peak on graph	N/A	Peak on graph	-0.01019
Baseline on graph	N/A	Baseline on graph	N/A	Baseline on graph	-0.01344
Difference	N/A	Difference	N/A	Difference	0.00325
Observed concen. (mg/L	0.0188	Obs. conc. (mg/L)	0.0103	Obs. conc. (mg/L)	0.0473
Final peak on graph	N/A	Final peak on graph	N/A	Final peak on graph	-0.01067
Difference	N/A	Difference	N/A	Difference	0.00277
Residual concen. (mg/L)	0.0188	Resid. conc. (mg/L)	0.0103	Resid. conc. (mg/L)	0.0403
Integrated Conc. (mg/L)	0.0188	Integ. Conc. (mg/L)	0.0103	Integ. Conc. (mg/L)	0.0437
C observed / C applied	1.00	C obs. / C appl.	1.00	C obs. / C appl.	0.99
Control Plate counts:		Control Plate counts:		Control Plate counts:	
413C Plate #1	60	414C Plate #1	138	415C Plate #1	74
413C Plate #2	53	414C Plate #2	134	415C Plate #2	78
413C Plate #3	52	414C Plate #3	131	415C Plate #3	60
Average	55.0	Average	134.3	Average	70.7
Standard Deviation	4.36	Standard Deviation	3.51	Standard Deviation	9.45
Variance	19.00	Variance	12.33	Variance	89.33
D^2	0.69	D^2	0.18	D^2	2.53
Within 90% Chi2 dist.	Yes	Within 90% Chi2 di	Yes	Within 90% Chi2 di	Yes
Dilution factor	1.00E+04	Dilution factor	1.00E+04	Dilution factor	1.00E+04
Experiment Plate Counts:		Experiment Plate Counts:		Experiment Plate Counts:	
213E Plate #1	36	214E Plate #1	83	015E Plate #1	152
213E Plate #2	35	214E Plate #2	72	015E Plate #2	194
213E Plate #3	39	214E Plate #3	99	015E Plate #3	185
Average	36.7	Average	84.7	Average	177.0
Standard Deviation	2.08	Standard Deviation	13.58	Standard Deviation	22.11
Variance	4.33	Variance	184.33	Variance	489.00
D^2	0.24	D^2	4.35	D^2	5.53
Within 90% Chi2 dist.	Yes	Within 90% Chi2 di	Yes	Within 90% Chi2 di	Yes
Dilution factor	1.00E+02	Dilution factor	1.00E+02	Dilution factor	1.00E+00
Log Kill:		Log Kill:		Log Kill:	
No	5.50E+05	No	1.34E+06	No	7.07E+05
N	3.67E+03	N	8.47E+03	N	1.77E+02
Log N/No	-2.18	Log N/No	-2.20	Log N/No	-3.60
Valid Data Point	Yes	Valid Data Point	Yes	Valid Data Point	Yes
CT	0.188	CT	0.134	CT	0.655

Experiment Number	DA030816	Experiment Number	DA030918	Experiment Number	DA030919
O3 conc. #1 (mg/L)	20.94	O3 conc. #1 (mg/L)	21.03	O3 conc. #1 (mg/L)	24.19
O3 conc. #2 (mg/L)	20.69	O3 conc. #2 (mg/L)	20.83	O3 conc. #2 (mg/L)	23.77
O3 conc. #3 (mg/L)	20.59	O3 conc. #3 (mg/L)	20.88	O3 conc. #3 (mg/L)	23.67
O3 conc. #4 (mg/L)	19.78	O3 conc. #4 (mg/L)	20.52	O3 conc. #4 (mg/L)	23.37
O3 conc. #5 (mg/L)	19.59	O3 conc. #5 (mg/L)	20.40	O3 conc. #5 (mg/L)	22.47
AVERAGE (mg/L)	20.32	AVERAGE (mg/L)	20.73	AVERAGE (mg/L)	23.49
Vol. of soln. (mL)	220.4	Vol. of soln. (mL)	220.1	Vol. of soln. (mL)	220.1
Vol. of stock O3 (mL)	0.079	Vol. of stock O3 (mL	0.082	Vol. of stock O3 (mL	0.425
Applied concen. (mg/L)	0.0073	Appl. conc. (mg/L)	0.0077	Appl. conc. (mg/L)	0.0454
Contact time (sec)	10	Contact time (sec)	10	Contact time (sec)	30
Peak on graph	N/A	Peak on graph	N/A	Peak on graph	0.00342
Baseline on graph	N/A	Baseline on graph	N/A	Baseline on graph	0.00061
Difference	N/A	Difference	N/A	Difference	0.00281
Observed concen. (mg/L	0.0073	Obs. conc. (mg/L)	0.0077	Obs. conc. (mg/L)	0.0409
Final peak on graph	N/A	Final peak on graph	N/A	Final peak on graph	0.00256
Difference	N/A	Difference	N/A	Difference	0.00195
Residual concen. (mg/L)	0.0073	Resid. conc. (mg/L)	0.0077	Resid. conc. (mg/L)	0.0284
Integrated Conc. (mg/L)	0.0073	Integ. Conc. (mg/L)	0.0077	Integ. Conc. (mg/L)	0.0341
C observed / C applied	1.00	C obs. / C appl.	1.00	C obs. / C appl.	0.90
Control Plate counts:		Control Plate counts:		Control Plate counts:	
416C Plate #1	63	418C Plate #1	89	419C Plate #1	64
416C Plate #2	52	418C Plate #2	105	419C Plate #2	70
416C Plate #3	57	418C Plate #3	110	419C Plate #3	56
Average	57.3	Average	101.3	Average	63.3
Standard Deviation	5.51	Standard Deviation	10.97	Standard Deviation	7.02
Variance	30.33	Variance	120.33	Variance	49.33
D^2	1.06	D^2	2.38	D^2	1.56
Within 90% Chi2 dist.	Yes	Within 90% Chi2 di	Yes	Within 90% Chi2 di	Yes
Dilution factor	1.00E+04	Dilution factor	1.00E+04	Dilution factor	1.00E+04
Experiment Plate Counts:		Experiment Plate Counts:		Experiment Plate Counts:	
216E Plate #1	69	218E Plate #1	79	019E Plate #1	156
216E Plate #2	61	218E Plate #2	69	019E Plate #2	177
216E Plate #3	55	218E Plate #3	69	019E Plate #3	211
Average	61.7	Average	72.3	Average	181.3
Standard Deviation	7.02	Standard Deviation	5.77	Standard Deviation	27.75
Variance	49.33	Variance	33.33	Variance	770.33
D^2	1.60	D^2	0.92	D^2	8.50
Within 90% Chi2 dist.	Yes	Within 90% Chi2 di	Yes	Within 90% Chi2 di	Yes
Dilution factor	1.00E+02	Dilution factor	1.00E+02	Dilution factor	1.00E+00
Log Kill:		Log Kill:		Log Kill:	
No	5.73E+05	No	1.01E+06	No	6.33E+05
N	6.17E+03	N	7.23E+03	N	1.81E+02
Log N/No	-1.97	Log N/No	-2.15	Log N/No	-3.54
Valid Data Point	Yes	Valid Data Point	Yes	Valid Data Point	Yes
CT	0.073	CT	0.077	CT	1.023

Experiment Number	DA032023	Experiment Number	DA033133	Experiment Number	DA033134
O3 conc. #1 (mg/L)	24.91	O3 conc. #1 (mg/L)	23.83	O3 conc. #1 (mg/L)	22.29
O3 conc. #2 (mg/L)	25.13	O3 conc. #2 (mg/L)	23.36	O3 conc. #2 (mg/L)	21.47
O3 conc. #3 (mg/L)	25.02	O3 conc. #3 (mg/L)	23.14	O3 conc. #3 (mg/L)	21.41
O3 conc. #4 (mg/L)	24.22	O3 conc. #4 (mg/L)	21.05	O3 conc. #4 (mg/L)	21.34
O3 conc. #5 (mg/L)	24.14	O3 conc. #5 (mg/L)	22.24	O3 conc. #5 (mg/L)	20.79
AVERAGE (mg/L)	24.68	AVERAGE (mg/L)	22.72	AVERAGE (mg/L)	21.46
Vol. of soln. (mL)	220.1	Vol. of soln. (mL)	220.1	Vol. of soln. (mL)	220.1
Vol. of stock O3 (mL)	1.104	Vol. of stock O3 (mL	1.212	Vol. of stock O3 (mL	0.469
Applied concen. (mg/L)	0.1232	Appl. conc. (mg/L)	0.1245	Appl. conc. (mg/L)	0.0456
Contact time (sec)	63	Contact time (sec)	64	Contact time (sec)	30
Peak on graph	0.00590	Peak on graph	-0.00494	Peak on graph	-0.00533
Baseline on graph	-0.00174	Baseline on graph	-0.01332	Baseline on graph	-0.00820
Difference	0.00764	Difference	0.00838	Difference	0.00287
Observed concen. (mg/L	0.1112	Obs. conc. (mg/L)	0.1220	Obs. conc. (mg/L)	0.0418
Final peak on graph	0.00459	Final peak on graph	-0.00632	Final peak on graph	-0.00577
Difference	0.00633	Difference	0.00700	Difference	0.00243
Residual concen. (mg/L)	0.0921	Resid. conc. (mg/L)	0.1019	Resid. conc. (mg/L)	0.0354
Integrated Conc. (mg/L)	0.1012	Integ. Conc. (mg/L)	0.1115	Integ. Conc. (mg/L)	0.0385
C observed / C applied	0.90	C obs. / C appl.	0.98	C obs. / C appl.	0.92
Control Plate counts:		Control Plate counts:		Control Plate counts:	
323C Plate #1	183	433C Plate #1	63	434C Plate #1	46
323C Plate #2	184	433C Plate #2	54	434C Plate #2	55
323C Plate #3	158	433C Plate #3	37	434C Plate #3	65
Average	175.0	Average	51.3	Average	55.3
Standard Deviation	14.73	Standard Deviation	13.20	Standard Deviation	9.50
Variance	217.00	Variance	174.33	Variance	90.33
D^2	2.48	D^2	6.79	D^2	3.27
Within 90% Chi2 dist.	Yes	Within 90% Chi2 di	Yes	Within 90% Chi2 di	Yes
Dilution factor	1.00E+03	Dilution factor	1.00E+04	Dilution factor	1.00E+04
Experiment Plate Counts:		Experiment Plate Counts:		Experiment Plate Counts:	
10mL23E Plate #1	35	10mL33E Plate #1	73	034E Plate #1	70
10mL23E Plate #2	38	10mL33E Plate #2	79	034E Plate #2	76
10mL23E Plate #3	31	10mL33E Plate #3	77	034E Plate #3	96
Average	34.7	Average	76.3	Average	80.7
Standard Deviation	3.51	Standard Deviation	3.06	Standard Deviation	13.61
Variance	12.33	Variance	9.33	Variance	185.33
D^2	0.71	D^2	0.24	D^2	4.60
Within 90% Chi2 dist.	Yes	Within 90% Chi2 di	Yes	Within 90% Chi2 di	Yes
Dilution factor	1.00E-01	Dilution factor	1.00E-01	Dilution factor	1.00E+00
Log Kill:		Log Kill:		Log Kill:	
No	1.75E+05	No	5.13E+05	No	5.53E+05
N	3.47E+00	N	7.63E+00	N	8.07E+01
Log N/No	-4.70	Log N/No	-4.83	Log N/No	-3.84
Valid Data Point	Yes	Valid Data Point	Yes	Valid Data Point	Yes
CT	6.397	CT	7.189	CT	1.154

Experiment Number	DA032427	Experiment Number	DA040536	Experiment Number	DA041040
O3 conc. #1 (mg/L)	22.60	O3 conc. #1 (mg/L)	23.31	O3 conc. #1 (mg/L)	23.70
O3 conc. #2 (mg/L)	22.02	O3 conc. #2 (mg/L)	23.36	O3 conc. #2 (mg/L)	23.28
O3 conc. #3 (mg/L)	22.14	O3 conc. #3 (mg/L)	22.85	O3 conc. #3 (mg/L)	23.38
O3 conc. #4 (mg/L)	22.00	O3 conc. #4 (mg/L)	22.92	O3 conc. #4 (mg/L)	22.63
O3 conc. #5 (mg/L)	21.43	O3 conc. #5 (mg/L)	22.44	O3 conc. #5 (mg/L)	22.37
AVERAGE (mg/L)	22.04	AVERAGE (mg/L)	22.98	AVERAGE (mg/L)	23.07
Vol. of soln. (mL)	220.57	Vol. of soln. (mL)	220.4	Vol. of soln. (mL)	220.41
Vol. of stock O3 (mL)	0.458	Vol. of stock O3 (mL)	0.439	Vol. of stock O3 (mL)	0.435
Applied concen. (mg/L)	0.0457	Appl. conc. (mg/L)	0.0457	Appl. conc. (mg/L)	0.0454
Contact time (sec)	30	Contact time (sec)	30	Contact time (sec)	30
Peak on graph	-0.00020	Peak on graph	-0.00659	Peak on graph	-0.00996
Baseline on graph	-0.00325	Baseline on graph	-0.00947	Baseline on graph	-0.01301
Difference	0.00305	Difference	0.00288	Difference	0.00305
Observed concen. (mg/L)	0.0444	Obs. conc. (mg/L)	0.0419	Obs. conc. (mg/L)	0.0444
Final peak on graph	-0.00124	Final peak on graph	-0.00688	Final peak on graph	-0.01039
Difference	0.00201	Difference	0.00259	Difference	0.00262
Residual concen. (mg/L)	0.0292	Resid. conc. (mg/L)	0.0377	Resid. conc. (mg/L)	0.0382
Integrated Conc. (mg/L)	0.0360	Integ. Conc. (mg/L)	0.0397	Integ. Conc. (mg/L)	0.0412
C observed / C applied	0.97	C obs. / C appl.	0.92	C obs. / C appl.	0.98
Control Plate counts:		Control Plate counts:		Control Plate counts:	
427C Plate #1	190	436C Plate #1	28	440C Plate #1	51
427C Plate #2	176	436C Plate #2	43	440C Plate #2	30
427C Plate #3	162	436C Plate #3	36	440C Plate #3	31
Average	176.0	Average	35.7	Average	37.3
Standard Deviation	14.00	Standard Deviation	7.51	Standard Deviation	11.85
Variance	196.00	Variance	56.33	Variance	140.33
D^2	2.23	D^2	3.16	D^2	7.52
Within 90% Chi2 dist.	Yes	Within 90% Chi2 dis	Yes	Within 90% Chi2 dis	Yes
Dilution factor	1.00E+04	Dilution factor	1.00E+04	Dilution factor	1.00E+04
Experiment Plate Counts:		Experiment Plate Counts:		Experiment Plate Counts:	
127E Plate #1	51	136E Plate #1	23	040E Plate #1	50
127E Plate #2	34	136E Plate #2	29	040E Plate #2	52
127E Plate #3	50	136E Plate #3	29	040E Plate #3	58
Average	45.0	136E Plate #4	27	040E Plate #4	78
Standard Deviation	9.54	Average	27.0	Average	59.5
Variance	91.00	Standard Deviation	2.83	Standard Deviation	12.79
D^2	4.04	Variance	8.00	Variance	163.67
Within 90% Chi2 dist.	Yes	D^2	0.89	D^2	8.25
Dilution factor	1.00E+01	Within 90% Chi2 dis	Yes	Within 90% Chi2 dis	Yes
		Dilution factor	1.00E+01	Dilution factor	1.00E+00
Log Kill:					
No	1.76E+06	Log Kill:		Log Kill:	
N	4.50E+02	No	3.57E+05	No	3.73E+05
Log N/No	-3.59	N	2.70E+02	N	5.95E+01
		Log N/No	-3.12	Log N/No	-3.80
Valid Data Point	Yes				
		Valid Data Point	Yes	Valid Data Point	Yes
CT	1.098				
		CT	1.189	CT	1.241

Experiment Number	DA041849	Experiment Number	DA041956	Experiment Number	DA041952
O3 conc. #1 (mg/L)	21.73	O3 conc. #1 (mg/L)	24.92	O3 conc. #1 (mg/L)	24.28
O3 conc. #2 (mg/L)	21.92	O3 conc. #2 (mg/L)	24.41	O3 conc. #2 (mg/L)	24.57
O3 conc. #3 (mg/L)	21.83	O3 conc. #3 (mg/L)	24.15	O3 conc. #3 (mg/L)	24.07
O3 conc. #4 (mg/L)	22.30	O3 conc. #4 (mg/L)	24.06	O3 conc. #4 (mg/L)	23.76
O3 conc. #5 (mg/L)	22.18	O3 conc. #5 (mg/L)	23.52	O3 conc. #5 (mg/L)	23.73
AVERAGE (mg/L)	21.99	AVERAGE (mg/L)	24.21	AVERAGE (mg/L)	24.08
Vol. of soln. (mL)	220.15	Vol. of soln. (mL)	220.15	Vol. of soln. (mL)	220.15
Vol. of stock O3 (mL)	1.064	Vol. of stock O3 (mL)	0.068	Vol. of stock O3 (mL)	0.069
Applied concen. (mg/L)	0.1058	Appl. conc. (mg/L)	0.0075	Appl. conc. (mg/L)	0.0075
Contact time (sec)	63	Contact time (sec)	10	Contact time (sec)	10
Peak on graph	0.00960	Peak on graph	N/A	Peak on graph	N/A
Baseline on graph	0.00240	Baseline on graph	N/A	Baseline on graph	N/A
Difference	0.00720	Difference	N/A	Difference	N/A
Observed concen. (mg/L)	0.1048	Obs. conc. (mg/L)	0.0075	Obs. conc. (mg/L)	0.0075
Final peak on graph	0.00851	Final peak on graph	N/A	Final peak on graph	N/A
Difference	0.00611	Difference	N/A	Difference	N/A
Residual concen. (mg/L)	0.0889	Resid. conc. (mg/L)	0.0075	Resid. conc. (mg/L)	0.0075
Integrated Conc. (mg/L)	0.0965	Integ. Conc. (mg/L)	0.0075	Integ. Conc. (mg/L)	0.0075
C observed / C applied	0.99	C obs. / C appl.	1.00	C obs. / C appl.	1.00
Control Plate counts:		Control Plate counts:		Control Plate counts:	
449C Plate #1	102	450C Plate #1	95	452C Plate #1	143
449C Plate #2	117	450C Plate #2	109	452C Plate #2	121
449C Plate #3	121	450C Plate #3	100	452C Plate #3	94
Average	113.3	Average	101.3	Average	119.3
Standard Deviation	10.02	Standard Deviation	7.09	Standard Deviation	24.54
Variance	100.33	Variance	50.33	Variance	602.33
D^2	1.77	D^2	0.99	D^2	10.09
Within 90% Chi2 dist.	Yes	Within 90% Chi2 dis	Yes	Within 95% Chi2 dis	Yes
Dilution factor	1.00E+04	Dilution factor	1.00E+04	Dilution factor	1.00E+04
Experiment Plate Counts:		Experiment Plate Counts:		Experiment Plate Counts:	
049E Plate #1	31	250E Plate #1	116	252E Plate #1	163
049E Plate #2	30	250E Plate #2	130	252E Plate #2	169
049E Plate #3	28	250E Plate #3	126	252E Plate #3	181
Average	29.7	250E Plate #4	102	252E Plate #4	176
Standard Deviation	1.53	Average	118.5	Average	172.3
Variance	2.33	Standard Deviation	12.48	Standard Deviation	7.89
D^2	0.16	Variance	155.67	Variance	62.25
Within 90% Chi2 dist.	Yes	D^2	3.94	D^2	1.08
Dilution factor	1.00E+00	Within 90% Chi2 dis	Yes	Within 90% Chi2 dis	Yes
		Dilution factor	1.00E+02	Dilution factor	1.00E+02
Log Kill:					
No	1.13E+06	Log Kill:		Log Kill:	
N	2.97E+01	No	1.01E+06	No	1.19E+06
Log N/No	-4.58	N	1.19E+04	N	1.72E+04
		Log N/No	-1.93	Log N/No	-1.84
Valid Data Point	Yes				
		Valid Data Point	Yes	Valid Data Point	Yes
CT	6.12				
		CT	0.076	CT	0.077

Experiment Number	DA042153	Experiment Number	DA042155	Experiment Number	DA062864
O3 conc. #1 (mg/L)	26.58	O3 conc. #1 (mg/L)	25.04	O3 conc. #1 (mg/L)	17.92
O3 conc. #2 (mg/L)	25.86	O3 conc. #2 (mg/L)	24.15	O3 conc. #2 (mg/L)	17.63
O3 conc. #3 (mg/L)	25.69	O3 conc. #3 (mg/L)	24.60	O3 conc. #3 (mg/L)	16.84
O3 conc. #4 (mg/L)	25.41	O3 conc. #4 (mg/L)	23.35	O3 conc. #4 (mg/L)	17.22
O3 conc. #5 (mg/L)	25.57	O3 conc. #5 (mg/L)	23.82	O3 conc. #5 (mg/L)	16.94
AVERAGE (mg/L)	25.82	AVERAGE (mg/L)	24.19	AVERAGE (mg/L)	17.31
Vol. of soln. (mL)	219.84	Vol. of soln. (mL)	219.84	Vol. of soln. (mL)	220.14
Vol. of stock O3 (mL)	0.889	Vol. of stock O3 (mL)	0.942	Vol. of stock O3 (mL)	0.957
Applied concen. (mg/L)	0.1040	Appl. conc. (mg/L)	0.1032	Appl. conc. (mg/L)	0.0749
Contact time (sec)	63	Contact time (sec)	63	Contact time (sec)	46
Peak on graph	0.00824	Peak on graph	0.00770	Peak on graph	0.01506
Baseline on graph	0.00123	Baseline on graph	0.00074	Baseline on graph	0.01000
Difference	0.00701	Difference	0.00696	Difference	0.00506
Observed concen. (mg/L)	0.1020	Obs. conc. (mg/L)	0.1012	Obs. conc. (mg/L)	0.0736
Final peak on graph	0.00717	Final peak on graph	0.00670	Final peak on graph	0.01402
Difference	0.00594	Difference	0.00596	Difference	0.00402
Residual concen. (mg/L)	0.0864	Resid. conc. (mg/L)	0.0867	Resid. conc. (mg/L)	0.0585
Integrated Conc. (mg/L)	0.0939	Integ. Conc. (mg/L)	0.0937	Integ. Conc. (mg/L)	0.0656
C observed / C applied	0.98	C obs. / C appl.	0.98	C obs. / C appl.	0.98
Control Plate counts:		Control Plate counts:		Control Plate counts:	
453C Plate #1	61	455C Plate #1	71	464C Plate #1	87
453C Plate #2	57	455C Plate #2	82	464C Plate #2	98
453C Plate #3	80	455C Plate #3	85	464C Plate #3	84
Average	66.0	Average	79.3	Average	89.7
Standard Deviation	12.29	Standard Deviation	7.37	Standard Deviation	7.37
Variance	151.00	Variance	54.33	Variance	54.33
D^2	4.58	D^2	1.37	D^2	1.21
Within 90% Chi2 dist.	Yes	Within 90% Chi2 dis	Yes	Within 90% Chi2 dis	Yes
Dilution factor	1.00E+04	Dilution factor	1.00E+04	Dilution factor	1.00E+04
Experiment Plate Counts:		Experiment Plate Counts:		Experiment Plate Counts:	
10mL53E Plate #1	55	055E Plate #1	39	10mL64E Plate #1	146
10mL53E Plate #2	44	055E Plate #2	34	10mL64E Plate #2	117
10mL53E Plate #3	41	055E Plate #3	29	10mL64E Plate #3	93
Average	46.7	055E Plate #4	27	10mL64E Plate #4	114
Standard Deviation	7.37	Average	32.3	Average	117.5
Variance	54.33	Standard Deviation	5.38	Standard Deviation	21.79
D^2	2.33	Variance	28.92	Variance	475.00
Within 90% Chi2 dist.	Yes	D^2	2.69	D^2	12.13
Dilution factor	1.00E-01	Within 90% Chi2 dis	Yes	Within 95% Chi2 dis	Yes
		Dilution factor	1.00E+00	Dilution factor	1.00E+00
Log Kill:					
No	6.60E+05	Log Kill:		Log Kill:	
N	4.67E+00	No	7.93E+05	No	8.97E+05
Log (N/No)	-5.15	N	3.23E+01	N	1.18E+02
		Log N/No	-4.39	Log N/No	-3.88
Valid Data Point	Yes				
		Valid Data Point	Yes	Valid Data Point	Yes
CT	5.951				
		CT	5.923	CT	3.010

Experiment Number	DA001
Effect of sodium formate	
Control Plate counts:	
001C Plate #1	62
001C Plate #2	77
001C Plate #3	91
Average	76.7
Standard Deviation	14.50
Variance	210.33
D^2	5.49
Within 99% Poisson dis	Yes
Dilution factor	1.00E+04
Experiment Plate Counts:	
001E Plate #1	72
001E Plate #2	64
001E Plate #3	74
Average	70.0
Standard Deviation	5.29
Variance	28.00
D^2	0.80
Within 99% Poisson dis	Yes
Dilution factor	1.00E+04
Log Kill:	
No	7.67E+05
N	7.00E+05
Log N/No	-0.04
Pooled Variance	
Sp^2 = ((m-1)s1^2 + (n-1)s2^2)/	
(m + n - 2)	119.17
Sp =	10.92
Test Statistic	
(x bar - y bar) / Sp /	
sqrt (1/m +1/n)	0.748
Degrees of Freedom	4
Table Value (alpha = 0.1;	
and t6,a/2)	+/- 1.533
Conclusion:	
Since 0.75 is betwee -1.533 and 1.533,	
the means are not significantly different	
even at the 90% confidence interval, an	
even less so at the 95 and 99% confidence	
intervals.	

Experiment Number	DA042456
O3 conc. #1 (mg/L)	24.39
O3 conc. #2 (mg/L)	23.67
O3 conc. #3 (mg/L)	23.17
O3 conc. #4 (mg/L)	23.08
O3 conc. #5 (mg/L)	23.36
AVERAGE (mg/L)	23.53
Vol. of soln. (mL)	223.70
Vol. of stock O3 (mL	0.428
Appl. conc. (mg/L)	0.0449
Contact time (sec)	30
Control Plate counts:	
656C Plate #1	28
656C Plate #2	17
656C Plate #3	29
Average	24.7
Standard Deviation	6.66
Variance	44.33
D^2	3.59
Within 90% Chi2 di	Yes
Dilution factor	1.00E+06
Experiment Plate Counts:	
456E Plate #1	32
456E Plate #2	33
456E Plate #3	42
456E Plate #4	38
Average	36.1
Standard Deviation	4.44
Variance	19.73
D^2	1.64
Within 90% Chi2 di	Yes
Dilution factor	1.00E+04
Log Kill:	
No	2.47E+07
N	3.61E+05
Log N/No	-1.83
Valid Data Point	Yes
CT	1.345

Experiment Number	DA042457
O3 conc. #1 (mg/L)	25.01
O3 conc. #2 (mg/L)	24.79
O3 conc. #3 (mg/L)	24.59
O3 conc. #4 (mg/L)	24.35
O3 conc. #5 (mg/L)	24.11
AVERAGE (mg/L)	24.57
Vol. of soln. (mL)	223.70
Vol. of stock O3 (mL	1.180
Appl. conc. (mg/L)	0.1290
Contact time (sec)	63
Control Plate counts:	
657C Plate #1	27
657C Plate #2	37
657C Plate #3	29
Average	31.0
Standard Deviation	5.29
Variance	28.00
D^2	1.81
Within 90% Chi2 di	Yes
Dilution factor	1.00E+06
Experiment Plate Counts:	
257E Plate #1	167
257E Plate #2	174
257E Plate #3	154
257E Plate #4	154
Average	162.3
Standard Deviation	9.95
Variance	98.92
D^2	1.83
Within 90% Chi2 di	Yes
Dilution factor	1.00E+02
Log Kill:	
No	3.10E+07
N	1.62E+04
Log N/No	-3.28
Valid Data Point	Yes
CT	8.170

Experiment Number	DA042658	Experiment Number	DA042660	Experiment Number	DA042862
O3 conc. #1 (mg/L)	24.85	O3 conc. #1 (mg/L)	23.92	O3 conc. #1 (mg/L)	23.18
O3 conc. #2 (mg/L)	24.03	O3 conc. #2 (mg/L)	24.25	O3 conc. #2 (mg/L)	23.09
O3 conc. #3 (mg/L)	24.04	O3 conc. #3 (mg/L)	23.56	O3 conc. #3 (mg/L)	22.66
O3 conc. #4 (mg/L)	23.52	O3 conc. #4 (mg/L)	22.98	O3 conc. #4 (mg/L)	22.36
O3 conc. #5 (mg/L)	23.05	O3 conc. #5 (mg/L)	22.86	O3 conc. #5 (mg/L)	22.25
AVERAGE (mg/L)	23.90	AVERAGE (mg/L)	23.51	AVERAGE (mg/L)	22.71
Vol. of soln. (mL)	224.30	Vol. of soln. (mL)	224.30	Vol. of soln. (mL)	224.00
Vol. of stock O3 (mL)	4.294	Vol. of stock O3 (mL	2.124	Vol. of stock O3 (mL	1.285
Applied concen. (mg/L)	0.4489	Appl. conc. (mg/L)	0.2206	Appl. conc. (mg/L)	0.1295
Contact time (sec)	180	Contact time (sec)	120	Contact time (sec)	63
Control Plate counts:		Control Plate counts:		Control Plate counts:	
658C Plate #1	24	660C Plate #1	25	662C Plate #1	38
658C Plate #2	21	660C Plate #2	22	662C Plate #2	39
658C Plate #3	27	660C Plate #3	36	662C Plate #3	34
Average	24.0	Average	27.7	Average	37.0
Standard Deviation	3.00	Standard Deviation	7.37	Standard Deviation	2.65
Variance	9.00	Variance	54.33	Variance	7.00
D^2	0.75	D^2	3.93	D^2	0.38
Within 90% Chi2 dist.	Yes	Within 90% Chi2 di	Yes	Within 90% Chi2 di	Yes
Dilution factor	1.00E+06	Dilution factor	1.00E+06	Dilution factor	1.00E+06
Experiment Plate Counts:		Experiment Plate Counts:		Experiment Plate Counts:	
158E Plate #1	26	260E Plate #1	23	262E Plate #1	88
158E Plate #2	29	260E Plate #2	17	262E Plate #2	83
158E Plate #3	23	260E Plate #3	28	262E Plate #3	75
158E Plate #4	28	Average	22.7	262E Plate #4	73
Average	26.5	Standard Deviation	5.51	Average	79.8
Standard Deviation	2.65	Variance	30.33	Standard Deviation	6.99
Variance	7.00	D^2	2.68	Variance	48.92
D^2	0.79	Within 90% Chi2 di	Yes	D^2	1.84
Within 90% Chi2 dist.	Yes	Dilution factor	1.00E+02	Within 90% Chi2 di	Yes
Dilution factor	1.00E+01			Dilution factor	1.00E+02
		Log Kill:			
Log Kill:		No	2.77E+07	Log Kill:	
No	2.40E+07	N	2.27E+03	No	3.70E+07
N	2.65E+02	Log N/No	-4.09	N	7.98E+03
Log N/No	-4.96			Log N/No	-3.67
		Valid Data Point	Yes		
Valid Data Point	Yes			Valid Data Point	Yes
		CT	0.469		
CT	80.956			CT	8.221