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University of Alberta

OZONE INACTIVATION OF *BACILLUS CEREUS* SPORES IN OZONE DEMAND FREE WATER

by

KEVIN MIN CHOE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

ENVIRONMENTAL SCIENCE

Department of CIVIL and ENVIRONMENTAL ENGINEERING

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Fall, 1998

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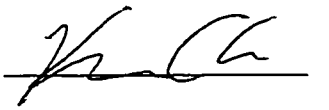

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled OZONE INACTIVATION OF *BACILLUS CEREUS* SPORES IN OZONE DEMAND FREE WATER submitted by KEVIN MIN CHOE in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in ENVIRONMENTAL SCIENCE.

Dr. G. R. Finch
Supervisor

Dr. S. J. Stanley
Examiner and Chair

Dr. M. Belosevic
Examiner

ABSTRACT

An in-depth analysis of ozone inactivation of aerobic spores of *B. cereus* was performed using bench-scale experiments with batch type reactor and pH 6 phosphate buffered ozone demand free water. The experiments were conducted at 22°C and 5°C. Incomplete gamma Hom models, which account for first-order disinfectant decay, were developed using the data collected. Generally, the inactivation level of spores was more sensitive to the change in ozone residual concentration than to the change in contact time. It was found that less contact time and/or ozone residual was required for up to 2 log inactivation at 22°C than at 5°C, but for 3 log removal same contact time and/or ozone residual was required at both temperatures. By comparing the inactivation kinetics of spores to a similar study on *C. parvum*, it was found that less contact time was required for up to 2 log inactivation of *B. cereus* spores using ozone residual of 1.0 mg/L. However, for inactivation of greater than 2 log, remarkably greater contact time was required for spores than the oocysts. This study showed that *B. cereus* spores are poor surrogates of ozone inactivation of *C. parvum* oocysts.

To my mother and father

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LIST OF FIGURES

LIST OF TABLES

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 INTRODUCTION

Public and technical interest has been focused on waterborne disease in the recent past due to several large-scale disease outbreaks caused by the parasitic protozoan, *Cryptosporidium parvum*. The number of people who have been infected in the largest ever documented outbreak of cryptosporidiosis, which occurred in the metropolis of Milwaukee in 1993, was reported to be some 400,000 (Fox and Lytle, 1994). Research surrounding the issue of *Cryptosporidium* in surface and treated waters has resulted in the collection of significant sets of information. Processes to reduce the number of *Cryptosporidium* in treated water to a safe level are still being developed.

Meanwhile, a proficient indicator may be necessary to assess the effect of the disinfectants that are being used against *Cryptosporidium*. Water quality indicators such as turbidity, particle counts, total/fecal coliform bacteria, and heterotrophic plate count (HPC) bacteria are being used to monitor the microbial quality of surface and treated water. The microbiology methods for most of the indicators are well established, less complex, more efficient, and less expensive than that of *Cryptosporidium*. However, the suitability of these organisms to serve as an indicator of very resistant pathogens such as *Cryptosporidium* is being questioned. Outbreaks of cryptosporidiosis in the various parts of the United States have been accounted for by suboptimal operating conditions in the

treatment plant. In several cases the water treated under these ill conditions still met the USEPA standards for allowable levels of turbidity and total coliform bacteria (Lisle and Rose, 1995).

Bacterial spores have been investigated in North America as a possible pathogen surrogate (Miltner *et al.*, 1997; Nieminski, 1997; Rice *et al.*, 1996). In general, the majority of *Bacillus* species are non-pathogenic saprophytes. They are primarily responsible for nutrient recycling in soil by virtue of their proteolytic and saccharolytic abilities. Only few species, including *B. cereus, B. subtilis* and *B. licheniformis,* have been reported to produce enterotoxins. Along with the advantage of low pathogenic properties, bacterial spores can be of importance as an indicator of pathogen removal. The relative ease of culturing and collecting the spores, well-defined laboratory techniques, and less expensive viability assessment after the disinfection procedures makes bacterial spores an ideal indicator for level of pathogen removal. Spores of the anaerobic bacterium, *Clostridium perfringens,* are being used with success in the Netherlands as a mandatory surrogate for protozoan cysts (Hijnen *et al.*, 1997).

Bacterial spores have been used for comparison with pathogens such as *Cryptosporidium* or have been included in the studies of indicator organisms in general (Miltner *et al.*, 1997; Nieminski, 1997; Rice *et al.*, 1996). An in-depth study of ozone on spores could not be found in the literature. While disinfection kinetic models have been established for organisms such as *E. coli, Giardia,* and *Cryptosporidium,* (Zhou and Smith, 1994; Finch *et al.*, 1994) such information

concerning bacterial spores could not be found in the literature. The spores have been recommended as a valuable indicator by some authors (Miltner *et al.*, 1997; Nieminski, 1997; Payment, 1991; Payment and Franco, 1993). To be of value, their disinfection kinetics should be comparable to that of the pathogen of concern. Also, the effect of temperature on ozone inactivation dynamics of spores remains to be explored.

This study was undertaken to develop a better understanding of ozone disinfection of the spores of *B. cereus*.

1.2 OBJECTIVES

The objectives of the present work were:

- to determine the ozonation requirements for different levels of spore inactivation at 22°C and 5°C;
- to develop kinetic models for ozone inactivation of *B. cereus* spores at 22°C and 5°C;
- to examine the effect of temperature on the inactivation of *B. cereus* spores by ozone;
- to compare the results with studies of ozone inactivation of *C. parvum* oocyst and other pathogens of concern; and
- to conclude on the adequacy of *B. cereus* spores as a disinfection surrogate for *Cryptosporidium* oocysts.

1.3 SCOPE OF STUDY

This study involved the collection of lab-cultured *B. cereus* spore (ATCC #14579) inactivation data using ozone at bench-scale. Experiments were performed in oxidant demand-free 0.05 M phosphate buffer at pH 6. The experiments were conducted at two temperatures to enable a comparison of the spore inactivation at room temperature and at low temperature. A disinfection kinetic model was developed for *Bacillus cereus* to enable a comparison to be made with a similar model for *Cryptosporidium* developed by others.

2. LITERATURE REVIEW

2.1 CHEMISTRY OF OZONE IN WATER

2.1.1 General

The usefulness of ozone as a disinfectant against pathogenic organisms as well as general treatment of water was known as early as 1886. Greater prevalence of water treatment plants utilizing ozone in Europe, especially in France and Germany is not surprising since the discovery of ozone's usefulness originated in those parts. Ozone as an alternative to chlorine in North America is becoming more popular, especially with increasing restrictions on the level of disinfectant by products (DBP) (Brink *et al.*, 1991). Ozone gas is an allotrope of oxygen, which has strong odor that can be detected at concentrations as low as 0.01 ppm (Kotz and Purcell, 1991). Some physical properties of ozone are shown in Table 2.1.

The most common method of ozone generation in water treatment is the use of corona discharge. The overall equation for ozone is as follows:

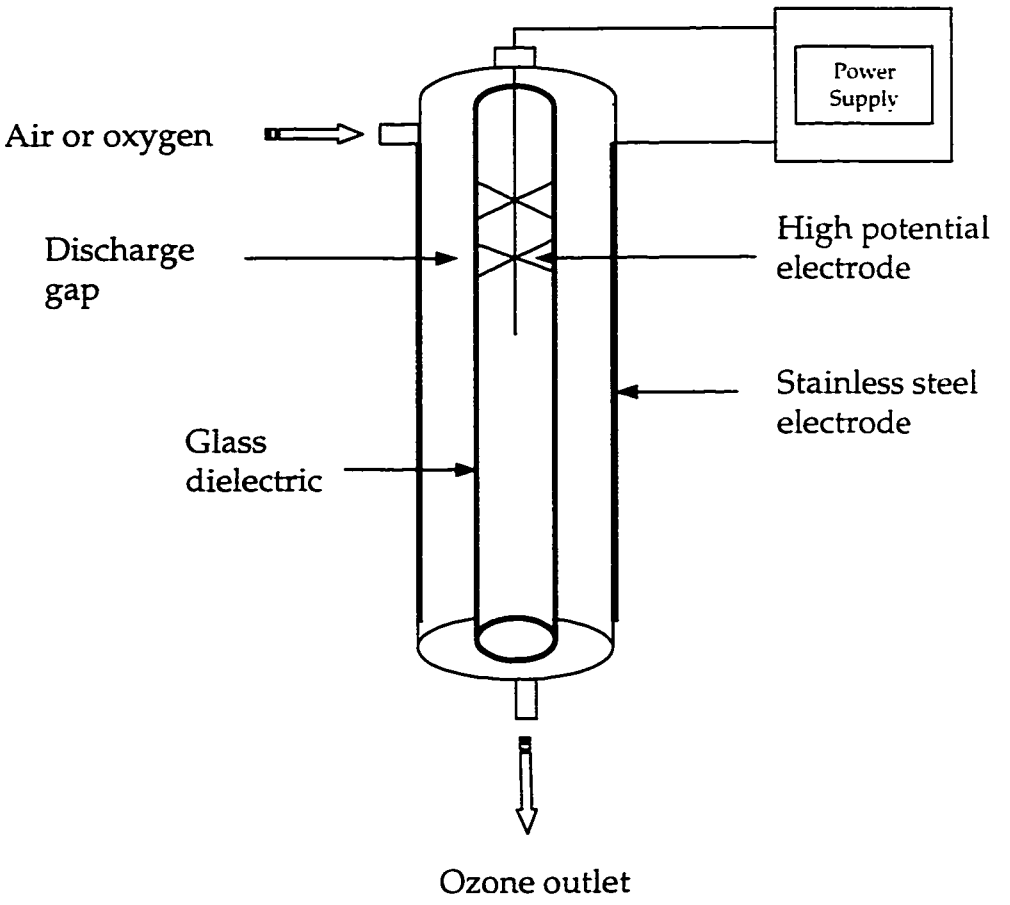
$$3O_2 + \text{energy} \rightarrow 2O_3 \qquad (2.1)$$

A schematic diagram of a corona discharge generator is represented by Figure 2.1. The principle components of the commercial grade generators are two metal

Table 2.1 Physical properties of ozone.

Properties	Values
Appearance	Colorless gas Dark blue liquid Blue-black crystalline
Molecular weight	47.998 g/mol
Density	Gas: 2.144 g/L Liquid: 1.614 g/L
Index of refraction	Liquid: 1.223
Melting point	-192.7 ± 2°C
Boiling point	-111.9°C

Source: after Kotz and Purcell (1991)



Source: after Bellamy *et al.* (1991)

Figure 2.1 Schematic diagram of a corona discharge ozone generator

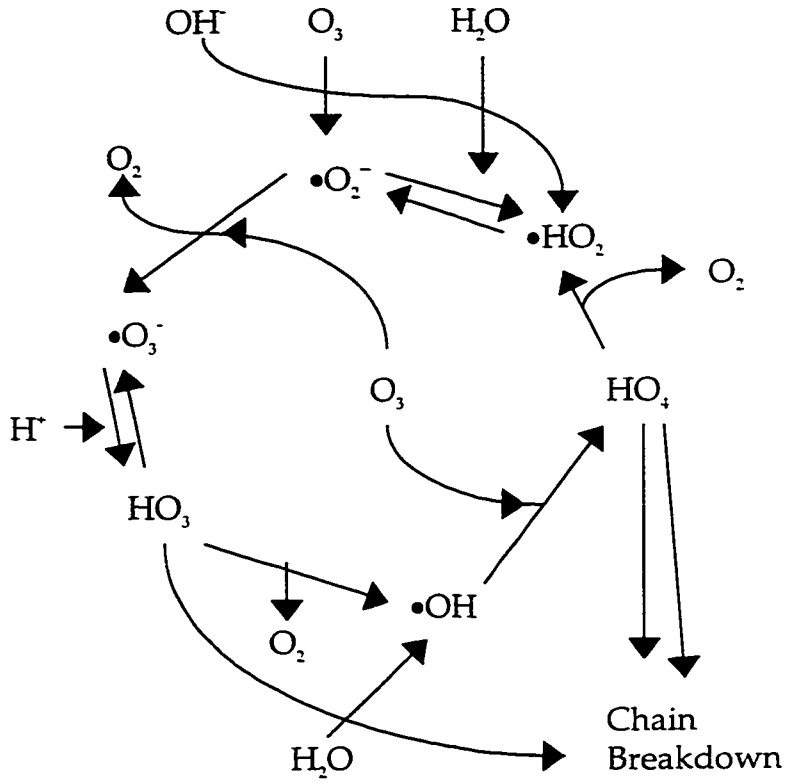
electrodes separated by a dielectric material. Applying high voltage to the electrode (1000 to 15,000 Volts depending upon the frequency) causes a corona discharge on the surface of the dielectric. The electrons travel back and forth between the two metal electrodes within the discharge gap at a frequency of 50 to 600 Hertz (Bellamy *et al.*, 1991). Some of the oxygen passing through the discharge gap gets ionized into free oxygen atoms, which then combine with molecular oxygen to form ozone. A dielectric is usually composed of specially formed glass or ceramic. It serves to dampen the energy in order to prevent sparking or arching between the two electrodes.

2.1.2 Decomposition of Ozone in Pure Water

Ozone is reactive regardless of whether it is in the gas phase or in the liquid phase. Once it is in aqueous solution, ozone undergoes a series of chain reactions decomposing back to molecular oxygen. The mechanism of ozone decomposition can be divided into three parts:

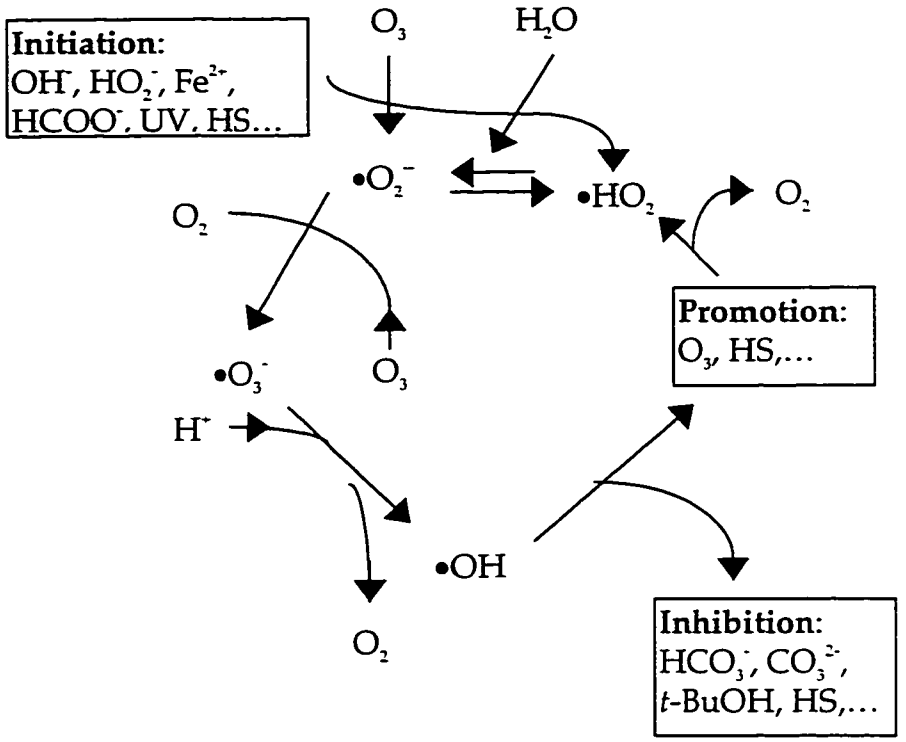
1. Initiation,
2. promotion, and
3. inhibition.

Two independent studies have proposed the ozone decomposition mechanism as a series of stepwise reactions. One is referred to as the Staehelin, Hoigné, and Bader mechanism (Staehelin and Hoigné, 1982) and the other is referred to as the Tomiyasu, Fukutomi, and Gordon mechanism (Tomiyasu *et al.*, 1985). The mechanisms are illustrated in Figure 2.2 and Figure 2.3, respectively.



Source: after Staehelin and Hoigné (1984)

Figure 2.2 Staehelin, Hoigné, and Bader decomposition reaction diagram



Source: after Bablon *et al.* (1991)

Figure 2.3 Tomiyasu, Fukutomi, and Gordon decomposition reaction diagram

The highly reactive radicals such as hydroxyl radical (•OH), superoxide radical ($\bullet O_2^-$), ozonide radical ($\bullet O_3^-$) and peroxide radical ($\bullet HO_2^-$) are mainly responsible for the decomposition of molecular ozone in ozone demand free (ODF) solutions. The rate constants for reactions involving these radicals with molecular ozone is in the neighborhood of 10^9 M^{-1} s^{-1} (Staehelin and Hoigné, 1985; Tomiyasu *et al.*, 1985).

Staehelin and Hoigné (1985) first put a pseudo first order rate law for ozone decomposition forth as follows;

$$-\left(d[O_3]/dt\right)_{pH} = k'_{OH^-}[O_3]^{1.0} \qquad (2.2)$$

Where k'_{OH^-} is the pseudo first order rate constant for a given pH value. This was possible only when hydroxyl radical scavengers such as bicarbonate and carbonate ions were introduced to the system to prevent secondary reactions from occurring. Tomiyasu *et al.* (1985) observed that in the absence of excess hydroxyl radical scavengers, the reaction order deviated from the pseudo first order and proposed the following rate law involving both first and second order terms:

$$-d[O_3]/dt = k_1[O_3] + k_2[O_3]^2 \qquad (2.3)$$

It has been noted that both k_1 and k_2 vary considerably with OH^- concentration and the presence or absence of radical scavengers (Tomiyasu *et al.*, 1985). At conditions where excess radical scavengers are present the second part of the rate law becomes unnecessary and the pseudo first order rate becomes prevalent.

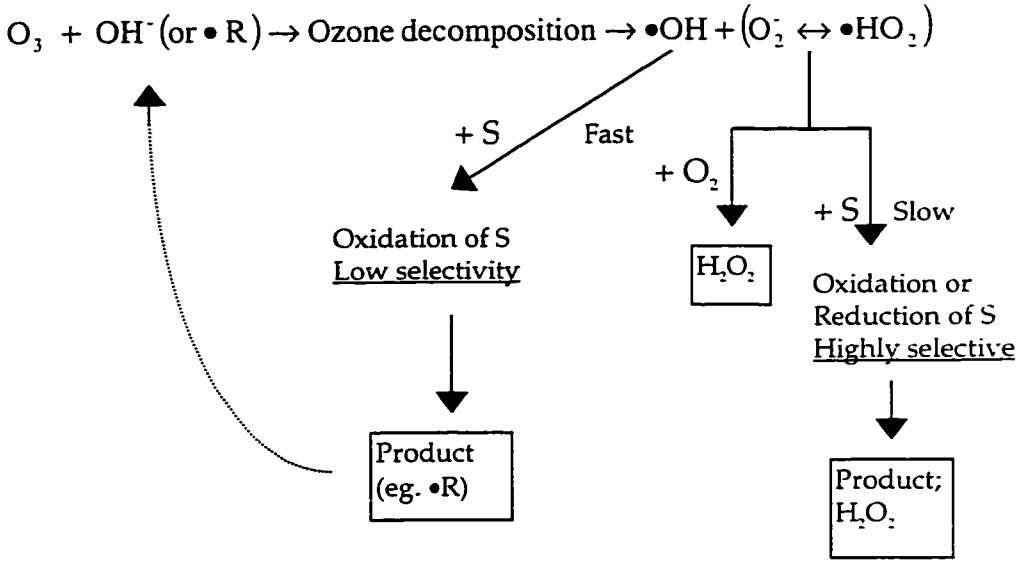
2.1.3 Decomposition of Ozone in Natural Water

In general, ozone may react with water impurities by means of direct or indirect reactions. Reaction between a substrate and ozone molecule is referred to as a direct reaction while the indirect reactions are referred to as those reactions which involve a substrate and radicals derived from decomposition of ozone. Ozone molecule is highly selective of species it will react with and when a reaction does take place it is relatively slow when compared with radical reactions. On the contrary, radicals are highly reactive and the reaction period is short. This can be best illustrated by Figure 2.4. Ozone can react with both organic and inorganic substances in natural waters. Some inorganic substrates that can be oxidized directly by ozone are Fe^{2+}, Mn^{2+}, Br^- and NH_3 (Bablon *et al.*, 1991a). Some organic substances that can be directly oxidized by ozone are alcohols, phenols and amines (Bablon *et al.*, 1991a). The reaction rate constants for most of these have been investigated in several papers (Hewes and Davidson, 1971; Hoigné and Bader, 1978; Staehelin and Hoigné, 1982; Staehelin and Hoigné, 1985).

Impurities naturally occurring in water contribute to initiation, promotion or inhibition of the ozone decomposition process. Initiators and promoters are

$$O_3 + S \xrightarrow{\text{slow}} \text{Direct oxidation of S} \xrightarrow{\text{Highly selective}} \text{Product}$$

or



Notes: S are the substrates or the impurities that will react with ozone and ozone radicals and R represents radicals.

Source: after Hoigné and Bader (1976)

Figure 2.4 Schematic illustration of direct and indirect reaction processes of ozone

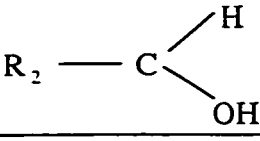
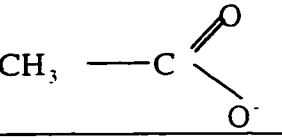
those substances that are capable of reacting with ozone or radicals that originated from ozone decomposition to form more radicals. Inhibitors are mainly those substances that react directly with ozone or ozone induced radicals and do not result in further generation of radicals. Table 2.2 lists some initiators, promoters and inhibitors of ozone decomposition by radical-type chain reaction that are found in natural water and wastewater. Total organic carbon (TOC) can either initiate or inhibit ozone decomposition. Organic compounds such as glyoxylic acid, formic acid, humic acid, primary alcohols and benzene have been found to initiate and promote ozone decomposition while acetone and aliphatic alcohols stop the decomposition chain (Hoigné and Bader, 1976; Staehelin and Hoigné, 1985). The cation species such as Fe^{2+} have been reported to initiate ozone decomposition (Bablon *et al.*, 1991b). By far the most effective agents for slowing down ozone decomposition are bicarbonate and carbonate ions. The rate constants measured for reaction of hydroxyl radicals with carbonate and bicarbonate are 2×10^7 $M^{-1}s^{-1}$ and 4×10^8 $M^{-1}s^{-1}$ (Staehelin and Hoigné, 1985). They can effectively scavenge the hydroxyl radicals and prevent further decomposition process.

2.2 INDICATORS OF DISINFECTION

2.2.1 General

Monitoring for human pathogens is difficult, time consuming and expensive to perform. Use of indicator organisms to monitor plant processes and to infer the

Table 2.2 Typical initiators, promoters, and inhibitors for decomposition of ozone by radical-type chain reaction, encountered in natural waters and wastewaters

Initiators	Promoters	Inhibitors
OH^{-1}		
H_2O_2/HO_2^-	Aryl – (R)	Alkyl – (R)
Fe^{2+}	Formate	HCO_3^-/CO_3^{2-}
Formate	Humics	Humics
Humics	O_3	TOC
TOC		

Notes: TOC and R represent Total Organic Carbon and radical respectively.

Source: after Staehelin and Hoigné (1985)

level of pathogens in both treated and untreated water is an important alternative to direct pathogen measurement. Careful and efficient use of well-defined surrogate indicator will lead to acceptable monitoring of disinfection processes. Certain qualities are deemed necessary for an organism to play an indicator role. They should (Grabow, 1990):

1. be present whenever pathogens are present;
2. ideally be present in greater numbers than pathogens if not at least present in the same number;
3. be at least as resistant to treatment processes as the pathogens;
4. preferably be non-pathogenic; and
5. be rapidly detectable using simple and inexpensive methods.

There is no indicator found to date that conforms to all of the above indicator qualities. In the light of recent waterborne disease outbreaks caused by encysted protozoa, common indicators currently in use should be reconsidered for their validity.

2.2.2 Bacterial Indicators

Several organisms are currently used as indicators of disinfection performance. Some of these are listed in Table 2.3 along with their definition, a species example, and their advantages and disadvantages as an indicator of disinfection processes. However, a significant correlation between the presence of the indicators in Table 2.3 and encysted protozoa removal could not be found anywhere in the literature.

Both anaerobic spores (*Clostridium*) and aerobic spores (*Bacillus*) have received some attention as possible indicators of encysted protozoa disinfection efficiency. The spores are reported to be found in all surface waters where encysted protozoa have been found (Chauret *et al.*, 1995; Payment and Franco, 1993; Rice *et al.*, 1996). There is a disagreement as to the correlation between the number of spores and the number of encysted protozoa but this is possible since both the level of spores and protozoa vary between water bodies and even within same water body (Chauret *et al.*, 1995; Rice *et al.*, 1996). However, spores correlate well with the numbers of other indicators throughout the water treatment processes. When *Clostridium* spores were monitored throughout conventional water treatment processes, it was found that there was a progressive reduction in both positive samples and actual numbers after each treatment process (Payment, 1991). In the Netherlands, spores of sulfite-reducing clostridia are used to monitor drinking water. The benefit of their use has been shown by their ubiquitous presence in surface water, their ability to accurately and consistently provide average removal for each process, their absence of re-growth, and their high correlation with protozoa removal (Hijnen *et al.*, 1997). Some of the other advantages of spores as an indicator of protozoan disinfection include:

1. relatively simple sampling, detection and viability analysis methods;
2. turnaround time is less than 24 hours;
3. inexpensive costs; and

Table 2.3 Common Bacterial Indicators

Type	Definition	Example	Advantages	Disadvantages
Coliform Bacteria	Those bacteria that are gram negative, non-spore forming, facultative rod that ferments lactose with gas formation within 48 hours at 35°C.	*Escherichia coli* *Klebsiella sp.* *Aerobacter sp.*	• Low-pathogenicity. • Simple, rapid and inexpensive methodology.	• Protozoans are present even when coliforms are undetectable. • Low resistance to disinfectants.
Coliphages	Those viruses that use coliform bacteria as the host for growth, multiplication, and propagation.	MS2 coliphage	• Quick analysis turn around time. • Adequate indicators of human virus.	• Analysis is more complex and expensive than coliforms. • Sensitive to some disinfectants.
Heterotrophic Bacteria	Those aerobic or facultatively anaerobic bacteria that use organic carbon as its principle energy source.	*Pseudomonas sp.* *Aeromonas. sp.* *Alcaligenes sp.* *Flavobacterium sp.*	• Simple, rapid and inexpensive methodology. • Present in higher numbers than pathogens. • Difficult to kill	• Lack of specificity. • Unable to correlate with any likelihood of waterborne disease outbreak.
Acid-fast Bacteria	Those bacteria that can not be easily decolorized after being stained with dyes such as fuchsin.	*Mycobacterium sp.*	• High resistance to disinfectants.	• Relatively long analysis turn around time. • Inaccurate counts.

4. high resistance to disinfectants.

Bacterial spores have been found to be more resistant to disinfectants than any of the known vegetative cells and viruses (Payment, 1991). More importantly the effect of common disinfectants, chlorine and ozone, on the spores have been tested and the results showed that spores are approximately equal in resistance when compared to encysted protozoa. Nieminski (1997) showed that there was a good correlation between removal of *Giardia* cysts and *Cryptosporidium* oocysts and the removal of total aerobic spores in water treatment processes with or without disinfection. Rice *et al.* (1996) reported that indigenous aerobic spores are more resistant to chlorine disinfection than *Giardia* cysts but are considerably less resistant than *Cryptosporidium* oocysts. Conversely, Miltner *et al.* (1997) found that indigenous endospores were more resistant to ozone than either of the protozoans. From a morphological perspective, spores and encysted protozoa both possess a thick-walled outer envelope, though of different structure, that protects the more sensitive inner contents. The outer envelope of a *Cryptosporidium* oocyst is composed of an inner and an outer wall with thickness of approximately 50 nm and 37 nm respectively (Reduker *et al.*, 1985). The outer envelope of a *Bacillus* spore consists of at least three layers: a cortex, a spore coat, and an exosporium. The combined thickness of the three outer layers of a *Bacillus* spore is approximately 200 nm (Gould, 1977). It is possible that these structures, which are lacking in vegetative forms, make them more resistant to disinfectants than the vegetative forms.

2.3 DESCRIPTION OF SPORULATED *B. CEREUS*

2.3.1 General Biology

Numerous genera of endospore forming bacteria are known. Groups with diverse backgrounds represent spores. Members of bacteria that are gram-positive or gram-negative, rod-shaped or cocci-shaped, strict aerobes or strict anaerobes, organic or inorganic substrate users and nitrogen fixers can produce spores. The genera that include spore-forming members are (Hobbs and Cross, 1983):

- *Bacillus*
- *Clostridium*
- *Desulfotomaculum*
- *Sporosarcina*
- *Sporolactobacillus*
- *Thermoactinomyces*

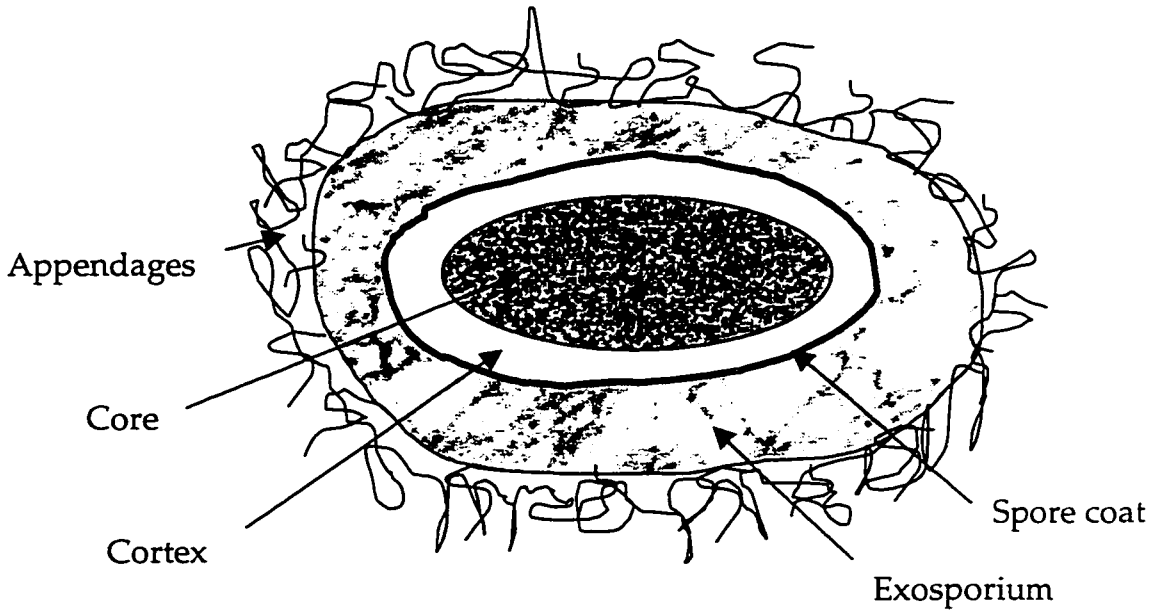
A common, widely distributed and commercially used spore is from the genus *Bacillus*. Harwood (1989) reported that genus *Bacillus* was first identified in the early nineteenth century by Ehrenberg and given the name *Bacillus* in 1872 by Ferdinand Cohn. Bacteria belonging to this group show a wide range of metabolic activities (Harwood, 1989). They are primarily heterotrophs deriving their energy from organic matter but there are also species that derive their energy from inorganic sources (*B. schlegelii*) (Schenk and Aragno, 1979). Some

are also known to fix nitrogen (*B. macerans* and *B. polymyxa*) (Hino and Wilson, 1958). The *Bacillus* group has the following characteristics:

- rod-shaped;
- form endospores;
- rods motile by means of peritrichously inserted flagella; and
- can occur in filaments (Buchanan, 1970).

The ability to form endospores by the members of the *Bacillus* genus was discovered independently by Ferdinand Cohn and Robert Koch in 1876. They have both found that at low nutrient conditions *Bacillus sp.* changed to a sporulated form that was found to be extremely resistant to heat. When the spores were put back in to a nutrient rich environment such as the intestinal tract of humans and animals, the spores germinated back to the vegetative form (Keynan and Sandler, 1983). *Bacillus* spores are ellipsoidal or spherical in shape and their approximate size is 0.5 x 1.0 x 2.0 microns (Rice *et al.*, 1996). A conceptual illustration of a *B. cereus* spore is shown in Figure 2.5.

The core is the innermost part of the spore and is surrounded by the inner membrane. It contains DNA, ribosome, cytoplasmic enzymes, dipiclonic enzymes along with calcium, which are required for germination, growth and reproduction at later stage of its lifecycle; a cortex mainly composed of peptidoglycan is the next layer followed by an outer membrane (or the coat); the



Source: after Husmark and Ronner (1992)

Figure 2.5 Conceptual illustration of the different layers of *B. cereus* spores

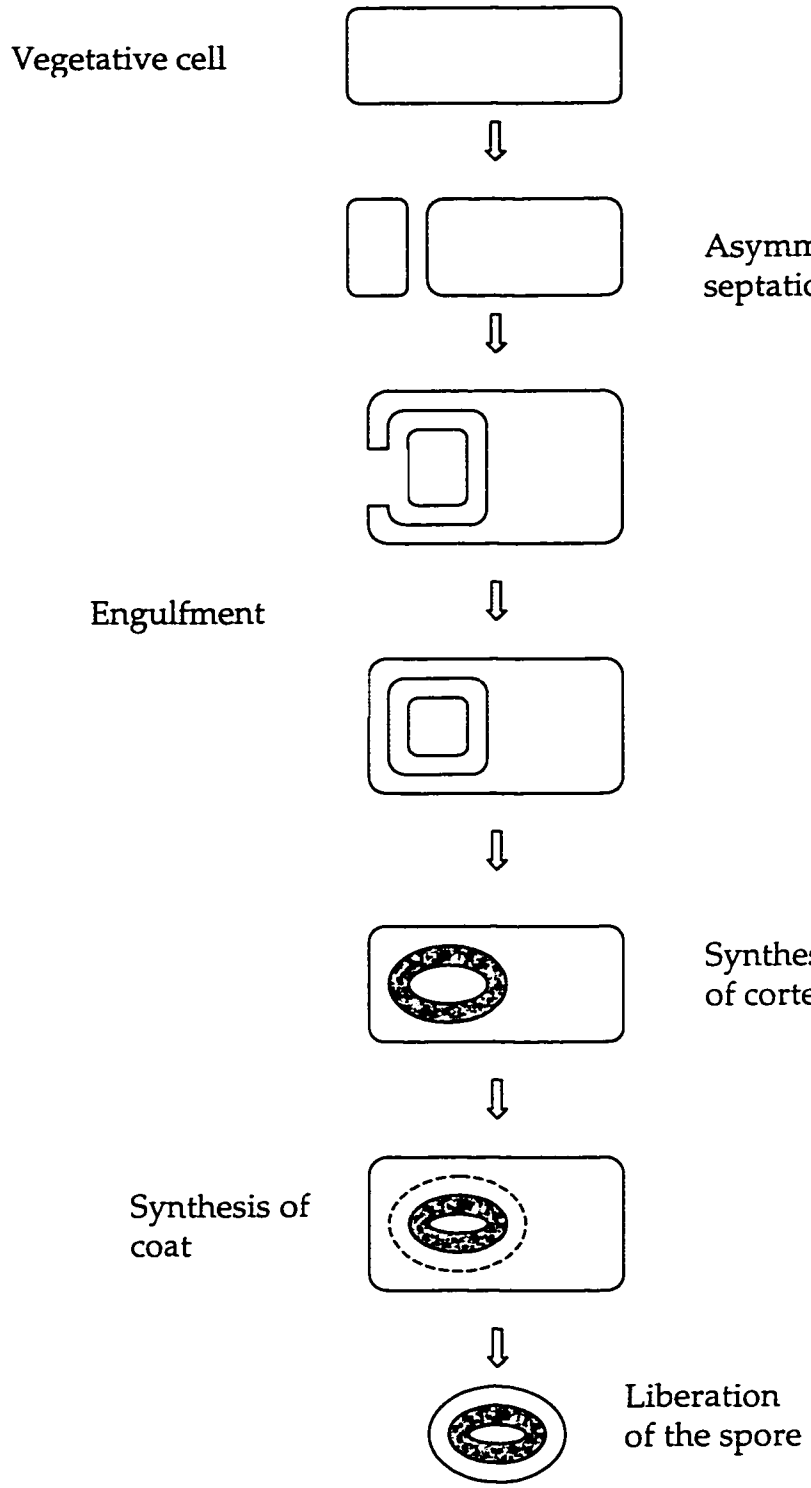
outer membrane is then coated with an enzyme resistant proteinaceous material known as the exosporidium (Gould, 1977). In the case of *B. cereus* spores, there are series of appendages on the surface of the exosporidium, which are used by the spores to adhere on to many surfaces.

2.3.2 Formation of the Bacterial Spores

Keynan and Sandler (1983) reported that life cycle of *Bacillus sp.* was first described in detail by Koch in 1876. A general illustration of the spore formation of *Bacillus cereus* is shown in Figure 2.6.

The sporulation process begins when the vegetative cell experiences a nutrient depletion in the surrounding media; the cell undergoes asymmetric septation in which one small and one large protoplast is created. A membrane surrounding the larger protoplast then engulfs the smaller protoplast; resulting in the isolation of a smaller protoplast surrounded by its own membranes and the membrane of the larger protoplast. The cortex is then synthesized between the two membranes and the sporulation process is complete by synthesis of the proteinaceous coat (Gould, 1977).

In general, bacteria are known to resort to two things when facing nutrient depression in their immediate surrounding area. They may change their intracellular and extracellular enzymes to feed on their own extracellular molecules that were expelled during the nutrient rich conditions (Nakata and Halvorson, 1960). They may also grow flagella, become chemotactically motile and go in search of other food sources (Ordal *et al.*, 1985).



Source: after Gould (1977)

Figure 2.6 Stages of sporulation involving major morphogenic changes

It should be emphasized that sporulation is the last step if all else fails. The end of a spore stage is reached when the spore senses abundance of nutrients surrounding it. The termination of the spore stage is marked by activation of the spores followed by germination and finally the outgrowth of the vegetative cell (Keynan and Sandler, 1983).

2.3.3 Occurrence of Spores

Bacterial spores are widespread because vegetative cells can perform a wide range of metabolic activities. Spore forming bacteria can be found in areas that are abundant in oxygen, low in oxygen, or absent in oxygen. They can use organic and inorganic substances as their food source. The spores which are highly resistant to extreme temperatures can be found in places where temperature is as high as 100°C or as low as 4°C. *B. cereus* has been found in soil, air, dust, water and in many raw and processed foods (Andersson *et al.*, 1995). They are especially abundant in dairy products due to the contamination of cow udders, which naturally come in contact with the soil (Te Giffel *et al.*, 1995). Other sources of spores from the food sector include meat, eggs, rice, spices and other dried foods (Kramer and Gilbert, 1989). Due to their saprophytic nature the spores are most abundant in soils. *Bacillus* spores have been isolated in soil samples from Antarctica to thermal springs and highly alkaline lakes (Harwood, 1989). The soil sample studies have substantially contributed to classifying spore-forming bacteria. Some spore-forming bacteria are classified in terms of their ability to resist extreme heat, extreme pH or low levels of organic or

inorganic contents in their living environment. It has been found in general that soils with low organic content restricts growth to few *Bacillus* species, including *B. cereus* (Harwood, 1989). As soil incurs more nutrients, the diversity of the bacteria increases. Due to its close association with the soil, water has harbored numerous spores. Some of the surface waters, including rivers and lakes, and groundwaters in the United States and Puerto Rico have been surveyed. The survey found anywhere between 100 to 10,000 colony forming units (cfu) per 100 mL (Rice *et al.*, 1996) for surface waters and less than 12 cfu/100 mL for ground waters (Rice *et al.*, 1996). The number of spores in surface waters increase by up to 20 times the normal count during the periods of heavy rainfalls and spring run-offs (Rice *et al.*, 1996).

2.3.4 Resistance of Spores to Chemicals

Earlier experiments using chlorine on *B. cereus* showed that only half of the spores were germinated on general growth media when exposed to 25 mg/L of free chlorine and germination was prevented completely when exposed to 50 mg/L (Wyatt and Waites, 1975). The exposure time for chlorine was not reported in the paper. Another interesting finding in the same study was that contact with chlorine significantly decreased the resistance of the spores to heat in *Clostridium bifermentans* and *Bacillus subtilis* but not for *B. cereus* (Wyatt and Waites, 1975). This is because germination of *B. cereus* spores is enhanced by heat activation. The germination stage involves three stages wherein the spore first loses its refractility, then swells and finally the spore elongates. Wyatt and

Waites' (1975) study showed that chlorine had the most effect on preventing the elongation of the germinated spore, less so on swelling and almost no effect on the loss of refractility. It can be inferred that chlorine inactivation is mainly by the reaction with the enzymes and molecules nearer the core of the spore, which are primarily responsible for the outgrowth of the vegetative cell upon germination.

Sagripanti and Bonifacino (1996) also looked at chlorine (in the form of 0.05 % sodium hypochlorite) inactivation of *Bacillus* spores and concluded that both pH and temperature had an effect. The pH effect was reported to be due to the variation in the level of active species of chlorine depending on the pH. Between pH of 5 and 7 more fraction of free chlorine is in the form of HOCl which is more efficient disinfectant than OCl^-. The spores suspended in water, without any disinfectants, were not affected by pH in the range of 2 to 12 (Sagripanti and Bonifacino, 1996). Three temperatures 8, 20 and 40°C were looked at in this study. It was found that a temperature effect on the inactivation rate of spores was only noticeable at 40°C and not between 8°C and 20°C.

In a more recent study by Rice *et al.* (1996), indigenous aerobic spores found in a local river water were treated with chlorine. The results of the study showed that for 2 and 3 log reduction a Ct value (a simple disinfectant residual concentration (mg/L) multiplied by the exposure time in minutes) of 114 mg•min/L and 315 mg•min/L was required respectively. They compared their results with the study by Williams and Russell (1991), who used pure laboratory

cultures of *B. subtilis* and concluded that a higher Ct value was necessary for the same inactivation in pure cultures because of the more uniform nature of pure cultures. The observed Ct value for 3 log reduction in the latter study was 500 mg•min/L (Williams and Russell, 1991).

Bacillus spores, like other microorganisms associated with drinking water, are more susceptible to ozone than to chlorine. A study by Korol *et al.* (1995) compared ozone and chlorine using spores of *B. subtilis* and reported up to 3.0 log inactivation with ozone but could not observe any considerable effect using chlorine at the same concentration and contact time used for ozone. A recent study by Miltner *et al.* (1997) showed that for a 2 log removal of indigenous spores a Ct (where C is the depth-averaged, dissolved ozone residuals) value near 19 mg•min/L was necessary.

The difficulty in studying the effect of disinfectants on spores lies in the fact that there are astonishingly large numbers of molecules associated with living organisms. Even relatively simple single-celled bacteria may possess millions of reactive sites for ozone and ozone induced radicals to react with. Some of the effects of chlorine on bacteria have been summarized in Table 2.4.

Few studies have looked at the activity of ozone with cellular constituents. Upon review of the *in vitro* studies of ozone reactions, Bablon *et al.* (1991a) concluded that ozone reacts relatively slowly with most carbohydrates and fatty acids while it is considerably more reactive to some amino acids and nucleobases. Bablon *et al.* (1991a) suggested that the preferred reaction sites of

ozone are the protein components of the cell wall and the membrane, and once ozone is inside the cytoplasm, chromosomes are the most susceptible targets.

Kulikovsky *et al.* (1975) and Wyatt and Waites (1975) have extensively examined the effects of disinfectants on spores. They both agree that the spore coat is the main barrier against disinfectants. The action of the NaOCl on *B. cereus* spore, as observed by electron microscope, occurred in series of sequential steps. Separation of the spore coat occurred first followed by changes in the electron density of the cortex and subsequent dissolving of the integument and finally the complete disruption of the spore. It has been shown that spore coats can be removed by using chemicals such as dithiothreitol, dithiothreitol with urea or urea with mercaptoethanol without preventing the spore from germinating (Aronson and Fitz-James, 1971). The spores with their coats removed in the above manner were up to 4000 times more susceptible to chlorine. When lysozyme, which has been shown to cause germination in spores with chemically removed spore coats (Aronson and Fitz-James, 1971), was added to the spores after the chlorine treatment there was no evidence of induced germination (Wyatt and Waites, 1975). Release and destruction of DNA accounted for 40% loss of DNA content in the spore after treatment with chlorine (Kulikovsky, 1975). Spore coats may be a significant barrier against disinfectants but the complete inactivation of the spore is not only due to the penetration of the disinfectant through the spore coat but further reaction of disinfectants with the contents of the core such as the DNA.

Table 2.4 The summary of the effect of chlorine on bacteria.

Target	Effects	Reference
Cell wall and membrane	• Permeability damage. • Release of cytoplasmic contents. • Increase in permeability to potassium.	(Kulikovsky, 1975) (Haas, 1976) " "
Cytoplasmic organelles	• Damage to cell nucleus.	(Chang, 1944)
Cell metabolism	• Inhibition of glucose oxidation in bacteria.	(Green and Stumpf, 1946)
Genetic material	• Inhibition of DNA synthesis. • DNA lesions.	(Haas, 1976) (Rosenkranz, 1973)

A study by Ishizaki *et al.* (1986) showed some indication that the effect of ozone on spore is similar to chlorine but at a much higher rate. When *B. subtilis* spores were exposed to ozone gas there was a initial lag phase followed by steep drop in the percent survival. The lag phase was interpreted as the time for the ozone gas to penetrate multiple barriers of the spore. The inactivation of the spore occurred after penetration of ozone in to the spore core. When there was high relative humidity on the surface of the spore ozone penetrated the spore barriers at a greater rate as evidenced by the significant decrease in the lag phase (Ishizaki *et al.*, 1986). The studies discussed above lead to the idea that success of ozone and chlorine disinfection of spores is dependent on how successfully they penetrate its multiple barriers to reach those vital sites.

2.4 KINETIC MODELLING OF DISINFECTION

Kinetic models of disinfection enable water treatment plants to efficiently control the process of disinfection. The kinetic modeling approach to disinfection originated from Chick (1908) who observed that a survival plot of anthrax spores disinfected with mercuric chloride resulted in a smooth curve. Chick observed a logarithmic function between the survival ratio and the exposure time and proposed the first disinfection kinetic equation. The equation is a first order unimolecular reaction describing the process of microbial disinfection. The concentration of reacting species were substituted with the numbers of surviving bacteria to state what is now referred to as the Chick's law (Chick, 1908):

$$-\frac{dN}{dt} = KN \quad \text{or} \quad \ln\left(\frac{N}{N_o}\right) = -Kt \tag{2.4}$$

Where t is the duration of organism's exposure to the disinfectant, N is the number of organism remaining at t, N_o is the initial number of organisms, and K is the inactivation rate constant. Chick asserted that the disinfectant, which reacts with the organism, is constant and ubiquitous in solution because it is present in enormously higher concentration relative to the organism.

Using Chick's data, Watson (1908) was able to show that the plot of log C (where C is the concentration of the disinfectant) versus log t always yielded a straight line. Thus he proposed the following simple and practical expression for a constant:

$$C^n * t = \text{a constant} \tag{2.5}$$

where C is the concentration of the disinfectant, n is a constant and t is the time of disinfection. Fair *et al.* (1948) adopted this empirical equation to describe the disinfection efficiency of chlorine. It was noted that when n is greater than one the efficiency of disinfectant decreases drastically with dilution of the disinfectant concentration. When n is less than one contact time in more important for inactivation and when n is equal to one the effect of concentration

and contact time is equal. Incorporation of Watson's empirical law into Chick's law yields the following pseudo first order rate law referred to as the Chick-Watson law:

$$-\frac{dN}{dt} = kNC^n \quad \text{or} \quad \ln\left(\frac{N}{N_o}\right) = -kC^n t \tag{2.6}$$

where N/N_o is the survival ratio for disinfectant concentration C after time t and k is the reaction rate constant.

In the Chick-Watson model, the rate of kill is solely dependent on the number of organisms remaining. The reactants are present in sufficient supply and as the number of organisms decline over time the rate also declines. Hom (1972) noted that disinfection of complex systems such as sewage pond effluents, which contain array of organisms including some multicellular forms, first order rate laws do not always apply and that contact time may play an important role. He referred to cases where the rate of kill increases with time (some multicellular organisms) and where the rate of kill decreases with time (natural die-off and resistance variation between different cells). Hom developed a m-order and n-order mathematical model as follows assuming chlorine as the disinfecting agent:

$$-\frac{dN}{dt} = mk'NC^n t^{m-1} \quad \text{or} \quad \ln\left(\frac{N}{N_o}\right) = -k'C^n t^m \tag{2.7}$$

where N/N_o is a survival ratio at concentration C and time t, k' is the reaction rate constant and n and m are constants for m-order and n-order reaction. When m is greater than 1 the shape of the curve for the plot of log number of organism versus time is convex yielding a "shoulder" effect, when m less than 1 the curve is convex yielding a "tail-off" effect. When m is equal to 1 the curve is a straight line resembling the pseudo first order Chick-Watson equation.

All of the three models described above assume that there is no decay in the disinfectants. Oxidants such as ozone have been shown to follow anywhere from half order to second order decay (Hewes and Davidson, 1971). Haas and Joffe (1994) reported that based on literatures describing the first order nature of chlorine and ozone decay the first order decay equation provides reasonable approximation for the decay of disinfectants:

$$C = C_o \exp(-k^* t) \tag{2.8}$$

where C_o is the initial disinfectant residual concentration and k^* is the first order decay rate. For the purposes of a more complete model for disinfection systems using oxidants that show significant level of decay, the first order decay equation has been incorporated into the Hom model resulting in (Haas and Joffe, 1994):

$$-\frac{dN}{dt} = mk' C_o^n \exp(-k^* tm) t^{m-1} N \quad \text{or} \quad \ln\left(\frac{N}{N_o}\right) = -mk' C_o^n \int_0^t \exp(-k^* tm) t^{m-1} dt \tag{2.9}$$

Haas and Joffe (1994) have showed that the above integration can be solved analytically by employing the Incomplete (γ) gamma function (Deming, 1944),

$$\gamma(\alpha, x) = \int_o^x \exp(-Z) Z^{\alpha-1} dz \tag{2.10}$$

where α greater than 0 and x greater than or equal to 0. The resulting equation, known as the Incomplete gamma Hom model, is then expressed as follows (Haas and Joffe, 1994):

$$\ln\left(\frac{N}{N_o}\right) = \frac{-mk' C_o^n}{(nk^*)^m} \bullet \gamma(m, nk^* t) \tag{2.11}$$

where m is greater than 0 and $nk^* t$ is greater than or equal to 0. The survival ratio can be calculated using any available computer software programs containing the table of the Incomplete gamma function.

3. MATERIALS AND METHODS

3.1 OXIDANT METHODS

3.1.1 Preparation of Ozone

The ozone protocol used in this study was previously described by Labatiuk *et al.* (1994). Ozone gas was produced by using a Welsbach laboratory corona discharge ozonator (model T-816, sunnyvale, CA) from extra dry oxygen gas (Praxair®, Mississauga, ON). The mixture of ozone and oxygen gas from the ozonator was continuously bubbled into a 500 mL gas absorption flask (Pyrex®, Corning, NY) containing approximately 300 mL of 22°C Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA) for 20 min..

3.1.2 Measurement of Ozone Concentration

A Pharmacia Biotech Ultrospec 2000 was used to measure stock ozone concentration before and after removal of specific volume from the reactor. The following formula was used to convert from absorbance unit to $[O_3]$ in mg/L:

$$0[O_3] = \frac{AMU}{k_{260}} * l * abs \qquad (3.1)$$

where AMU is the atomic mass unit for ozone which is 48000 mg/mol, k_{260} is molar absorptivity for ozone (3300 $mol^{-1}cm^{-1}$) (Hart *et al*, 1983), l is length of the light passage through the cuvette which is 10 mm, *abs* is absorbance at 260 nm.

The concentration of the stock was calculated to be between 18 and 21 mg/L. The ozone concentration in the reactor during the inactivation experiments was continuously monitored in a continuous flow cell throughout the contact time by diode-array spectrophotometer (Hewlett-Packard Canada, Ltd., Toronto, Ontario, model 8452A).

3.2 MATERIALS

3.2.1 Phosphate Buffer Solution

All experiments were performed in pH 6 ozone demand free phosphate buffer solution (0.05M). Buffer solution was prepared in 4 L batches. 3.476 g of Na_2HPO_4 (AnalaR, BDH, Poole, England) and 27.272 g of KH_2PO_4 (AnalaR, BDH, Poole, England) were measured out and dissolved to make 4 L solution with Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA). The solution was then ozonated for 40 min.. It was left standing, slightly capped with aluminum foil, overnight. The next day it was boiled for at least 20 min. to remove any ozone. After, the solution cooled to room temperature and the pH was confirmed.

3.2.2 Glassware

Glassware were prepared using Fisher®-Scientific Lab Washer (model 97-975, Pittsburgh, PA). The wash consisted of five cycles. 1) pre-wash, 2) main wash using Fisherbrand® Sparkleen™ (Pittsburgh, PA) detergent, 3) phosphoric acid (20 to 30 %) rinse 4) final rinse and 5) drying with heating. The water

supplied to the washer was distilled and deionized. During the main wash and final rinse the water temperature were 75°C and 70°C respectively. All the water used by the washer was softened using a water softener (Somat Salt) to guard against any hardness. After the wash, all of the glassware were immediately covered with aluminum foil and oven dried at 100°C (Fisher Scientific Isotemp® 500 series, Pittsburgh, PA) overnight. They were removed the following day and placed in the cabinet ready to be used.

3.2.3 Ozone Demand Free Glassware

Ozone demand free glassware was initially prepared the same as non-ozone demand free glassware. After they were removed from the washer they were capped with aluminum foil and put under the fume hood. After that they were filled with ozonated water with ozone residual concentration of approximately 20 mg/L to the brim (overflow). It was left standing overnight. The following day ozonated water was poured out, glassware recapped with aluminum foil and oven-dried (100°C) for at least 3 hours. A yellow sticker was placed on the cap to indicate that the glassware was ozone demand free.

3.3 MICROBIOLOGY METHODS

3.3.1 Organism

Spores of *B. cereus* vegetative cell of the American type culture collection (ATCC #14579) were used. They were obtained from the Food Microbiology department at the University of Alberta.

3.3.2 Nutrient Agar

Nutrient agar was prepared according to the manufacturer's instructions (Difco, Detroit, MI). Approximately 23 g of nutrient agar powder was dissolved in 1000 mL of Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA). The resulting solution was then mixed over a hot plate stirrer (Corning, NY) until the molten agar solution was formed. While in molten phase, roughly 14 mL of agar was siphoned using 10 mL sterile glass pipette (FisherBrand® Fisher Scientific, Pittsburgh, PA) and placed in a clean glass 16 x 125 mm test tube (FisherBrand® Fisher Scientific, Pittsburgh, PA) and capped with a plastic cap. The deeps (test tubes containing the molten agar) were then autoclaved at 121°C for 25 minutes. The deeps were allowed to solidify and then stored in a cabinet until required. The agar in the deep was melted by hot water bath submersion (approximately 80°C) before being poured on to a 100 x 15 mm sterile polypropylene Petri dishes (FisherScientific, Pittsburg, PA).

3.3.3 Nutrient Broth

Nutrient broth was prepared for rejuvenation of the *B. cereus* spores. Approximately 8 g of BBL® nutrient powder (Beckton Dickinson, Meylan, France) was dissolved in one liter of Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA). The solution was warmed slightly to secure a homogenous state. Then, approximately 10 mL pipette samples was taken and transferred to clean glass 16 x 125 mm test tubes (FisherBrand® Fisher Scientific, Pittsburgh, PA) which were then capped and autoclaved at 121°C for 25 minutes.

The final solution contained 5 g of pancreatic digest of gelatin and 3 g of beef extract per liter with a pH of 6.9±0.2. The test tubes containing sterile BBL nutrient broth were stored in a refrigerator at 4°C until use.

3.3.4 GBBM Medium

GBBM media was prepared for the purpose of sporulating the vegetative cells of *B. cereus*. The medium was prepared by combining together BBM medium, Grelet salts solution and calcium chloride solution. BBM medium was composed of (per 120 mL of Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA)): 0.60 g protease peptone #2 (Difco, Detroit, MI), 0.60 g protease peptone #3 (Difco, Detroit, MI), and 2.16 g Nutrient Agar (Difco, Detroit, MI). The Grelet salts solution was composed of (per 520 mL of Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA)): 5.2 g potassium nitrate, 0.88 g potassium dihydrogen orthophosphate, 90 mg potassium sulfate, 31 mg magnesium sulfate, 1 mg manganese sulfate, 3 mg ferrous sulfate, 7 mg zinc sulfate and granulated agar (1.2 to 1.5 % per total volume of GBBM medium). The final pH of the Grelet salts solution was adjusted to 6.9 using solid potassium hydroxide. Calcium chloride solution was prepared by dissolving 1.17 g of calcium chloride into 80 mL of Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA). Each of the above preparations was autoclaved at 121°C for 25 min. before being combined. They were then poured in to 100 x 15 mm sterile polypropylene Petri dishes (FisherScientific, Pittsburg, PA) and stored at 4°C until use.

3.3.5 Dilution Water

The dilution water was prepared by following the procedure outlined in the Standard methods (APHA, 1992). Dilution water was prepared 4 L at a time. 34.0 g of potassium dihydrogen orthophosphate was dissolved in 500 mL of Elga® water. The pH of this solution was adjusted to 7.2 ±0.5 with 1 N sodium hydroxide. It was then diluted to 1 L with Elga® water. Salt solution was prepared by dissolving 81.1 g of magnesium chloride in 1 L of Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA). 5 mL of stock phosphate buffer solution and 20 mL of magnesium chloride solution was added to 4 L of Elga® water (Elga® Maxima ultrapure water, Pittsburgh, PA). They were dispensed in to milk bottles in required amounts and autoclaved for 20 min. at 121°C.

3.3.6 Rejuvenation of the Spores

200 µL of original suspension was removed and placed into 10 mL of sterile BBL nutrient broth solution (Beckton Dickinson, Meylan, France) in a glass 16 x 125 mm test tube. A test tube containing only the broth was used as a control. They were left to stand at room temperature for 2 to 3 days until the suspension became cloudy with vegetative cells in the tube seeded with spores. The control retained the clear yellow broth suspension.

3.3.7 Sporulation of the Vegetative Cells

Once the vegetative cells were ready, 100 µL of the broth suspension was removed and plated on the GBBM medium. 25 plates were plated in this manner and incubated (FisherScientific Isotemp® incubator, Pittsburgh, PA) at 37°C for at least 5 days and no more than 7 days.

3.3.8 Washing and Purification of the Spores

Plates were taken out of the incubator and spores were removed from the media surface by gently scraping with flame-sterilized bent glass rod. The spores were collected in a sterile 50 mL screw cap conical tube (Starstedt, Newton, NC) containing 30 mL of pH 6 ozone demand free phosphate buffer. Spores were washed 8 times with pH 6 ozone demand free phosphate buffer by centrifugation at 500 x *g* for 10 minutes (Jouan® centrifuge CR 4.22, rotation radius = 185 mm, M4 swing-out rotor). After the eighth wash the suspension was vortexed and submerged in a 80°C water bath for 20 minutes to kill off any vegetative cells. It was washed again 3 times by centrifugation. The final wash was stored at 4°C.

3.4 INACTIVATION PROCEDURES

3.4.1 Room Temperature Experiments

For each trial, two 250 mL Erlenmeyer flask reactors were filled with 200 mL of pH 6 ozone demand free phosphate buffer, and had approximately 5 x 10^5/mL *B. cereus* spores added. Each reactor was completely covered with

aluminum foil to protect it from UV radiation. Immediately after the spores were added to the water, the solution was stirred at high intensity for 30 seconds using a teflon–coated magnetic stir bar (Thermix® model 220T, FisherScientific, Pittsburgh, PA). The suspension was then left at low stirring throughout at least 30 min. of acclimatization period and throughout the experimental run. An ozone concentration of between 0.4 to 2.0 mg/L was added to one of the reactors. No ozone was added to the second reactor that served as a control. Instead an equivalent volume of ozone demand free water was added. Both of the reactor contents were continuously stirred throughout the contact time by using a stirrer. At the end of the desired contact time, 400 µL of 1 M sodium formate was added to each of the reactors to remove any remaining ozone.

3.4.2 Cold Temperature Experiments

The procedures were identical to room temperature experiments. The cold temperature apparatus consisted of a shaker bath (Lab-Line Instruments Inc., Mel Rose Park, IL) with recirculating cooler (FTS® Systems Inc., Stone Ridge, NY). Before the spores were added, the reactor solution was shaken in the bath until the solution temperature reached 5°C. Then the spores were added to the reactors and shaken in the bath for at least another 30 minutes for the spores to be acclimatized. Then, the experimental reactor was taken out of the shaker bath and put in to a Styrofoam lined ice cream pail. The ice cream pail was placed on a stirrer (Thermix® model 220T, FisherScientific, Pittsburgh, PA). The pail had two hoses attached to the side, one near the bottom and one near

the top. A submersible pump (Beckett Corporation, Dallas, TX) was pumping the 5°C water from the shaker bath into the pail via the bottom tube. The water emptied from the pail by means of the upper tube. Thus the pail was being constantly replenished with 5°C water from the shaker bath. The control reactor was left shaking in the water bath throughout the course of the experiment.

3.5 SAMPLING AND VIABILITY ASSAY

At the end of the contact time, the aluminum foil was removed and the contents were kept at a low stir then stopped. A 10 mL sample from the ozone reactor and the control reactor was taken and put in to milk dilution bottles that were pre-filled with 90 mL of sterile dilution water. The dilutions of up to 10^{-4} were made for both of the samples. A slightly modified Pour plate method described in standard methods (APHA, 1992) was used in order to minimize the individual colony size for more accurate plate count. Three samples of 1 mL from each dilution and control were put into center of inside bottom of three Petri dishes. A deep (a test tube containing 15 mL of molten nutrient agar (Difco, Detroit, MI) between 60-90°C) was then poured directly over the 1 mL sample. The contents of the plates were then swirled for even distribution of the spores. For each set of experimental trials two sets of three control plates were made. Three control plates contained only the molten agar while the other three contained molten agar plus 1 mL of blank dilution water. All plates were incubated at 37°C for 20 to 24 hours. Only the viable counts within the range of 20 to 300 were used. Fisher's D^2 statistic was used to test whether the differences

observed between the replicates were Poisson or not. The χ^2 distribution was used as a reference distribution. The equation for the Fisher's D^2 statistic is (Haas and Heller, 1986):

$$D^2 = \frac{(n-1)s^2}{\bar{x}} \qquad (3.2)$$

where n is the number of replicates, s^2 is the variance and the x is the mean. Fisher's D^2 statistic was calculated for each of the dilutions with three plates that produced colonies within the 20 to 300 ranges. The values were compared against the χ^2 distribution for α equal to 0.05 and 2 degrees of freedom. The critical χ^2 value for α equal to 0.05 was found to be 5.991 (Miller *et al.*, 1990). Only the average of the counts from the three plates that had D^2 values less than 5.991 were used. The average of the counts from the three plates that had D^2 values exceeding 5.991 were rejected as inaccurate counts. The spore concentration of the reactor was then back calculated from the dilution plate counts and used as the estimated value of the real concentration. If replicates from two or more dilutions had counts within the range of 20 to 300 and D^2 values less than 5.991 than the average of the two estimated values of the spore concentration in the reactor was taken. The concentration derived in this manner for the control reactor was used as an estimate of N_o and the concentration of the experimental reactor was used as N. The following formula was used to calculate the percent inactivation and log inactivation:

$$\% \text{ inactivation} = 100 - \left(N / N_o * 100 \right) \tag{3.3}$$

$$\log \text{ inactivation} = - \log \left(\frac{N}{N_o} \right) \tag{3.4}$$

where N is concentration of spores remaining after ozone treatment and N_o is concentration of spores before treatment of ozone.

4. RESULTS AND ANALYSIS

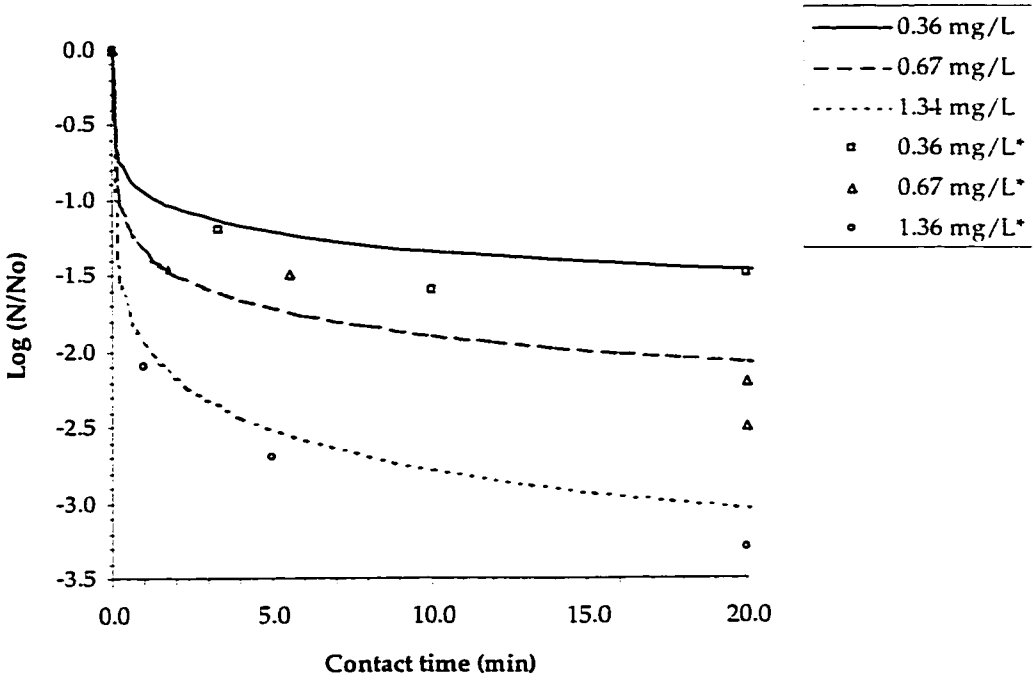
4.1 INACTIVATION DATA

Thirteen experimental trials were performed at room temperature. The temperature of the room varied from 20.5°C to 22.5°C. The initial oxidant residual and contact time ranges used were 0.4 mg/L to 1.55 mg/L and 1 minute to 20 minutes. Ten experimental trials were performed in cold temperature water bath. The temperature of the water bath varied from 4.8°C to 5.2°C. The initial oxidant residual and contact time ranges used were 0.35 mg/L to 1.71 mg/L and 5 minutes to 20 minutes. The results from these experiments are summarized in Table 4.1. A typical survival plots of ozone inactivation in a batch reactor is shown by Figure 4.1. The smooth curve on Figure 4.1 was generated using the IgH kinetic model. The scatter plots represent the observed data. The ozone residual shown in Figure 4.1 is defined as the average of the initial and the final residual measurements.

When low initial ozone residual was used (less than 0.5 mg/L) log inactivation appeared to be affected by an increase in contact time initially (at less than 5 minutes) but did not change as contact time progressed (greater than 6 minutes). Log inactivation versus contact time for an initial ozone residual of around 0.4 mg/L was plotted (see Figure 4.2). There was a rapid initial removal stage (within 5 minutes of contact time) followed by a "tailing-off" of removal as contact time increased beyond 5 minutes.

Table 4.1 Summary of the *B. cereus* spore inactivation experiments performed at pH 6 ozone demand free phosphate buffered water using ozone as a disinfectant

Experiment number	Temperature (0C)	Initial Concentration (mg/L)	Final Concentration (mg/L)	Time duration of disinfection (min.)	Decay rate of disinfectant (estimated by first order model)	Detected log removal (log(N/No))	Incomplete gama Hom predicted log removal (log(N/No))
E42	22.0	0.5	0.3	10.4	0.048	-1.6	-1.5
E44	22.0	0.9	0.5	15.1	0.045	-2.7	-2.3
E45	22.0	0.5	0.4	5.4	0.045	-1.5	-1.5
E47	22.0	1.5	1.2	5.0	0.043	-2.7	-2.7
E48	22.0	1.1	0.6	18.0	0.036	-2.3	-2.6
E49	22.0	0.9	0.4	20.1	0.041	-2.2	-2.4
E50	22.0	0.4	0.2	20.0	0.035	-1.5	-1.6
E51	22.0	0.9	0.4	19.7	0.036	-2.5	-2.3
E52	22.0	1.6	0.9	19.5	0.031	-3.3	-3.2
E54	22.0	1.3	0.6	20.0	0.034	-2.8	-2.9
E56	22.0	0.4	0.4	3.3	0.032	-1.2	-1.2
E57	22.0	0.5	0.5	1.1	0.038	-1.0	-1.1
E58	22.0	1.5	1.4	1.1	0.033	-2.1	-2.1
E23	5.0	0.4	0.3	5.5	0.065	-1.0	-0.8
E31	5.0	0.4	0.3	5.0	0.049	-0.6	-0.8
E32	5.0	0.4	0.2	19.9	0.026	-1.2	-1.1
E33	5.0	1.7	1.5	5.2	0.032	-2.2	-2.0
E34	5.0	1.1	0.6	19.9	0.032	-2.3	-2.2
E38	5.0	0.6	0.5	5.6	0.030	-0.8	-1.0
E41	5.0	0.5	0.3	10.1	0.028	-1.1	-1.0
E46	5.0	0.9	0.6	15.0	0.025	-2.0	-1.8
E53	5.0	1.7	1.3	20.1	0.013	-3.1	-3.1
E55	5.0	1.4	1.0	19.9	0.015	-2.9	-2.7



Notes: Data is for 22°C.

*Observed data points.

Figure 4.1 Typical survival curves of ozone inactivation of *B. cereus* spores at pH6

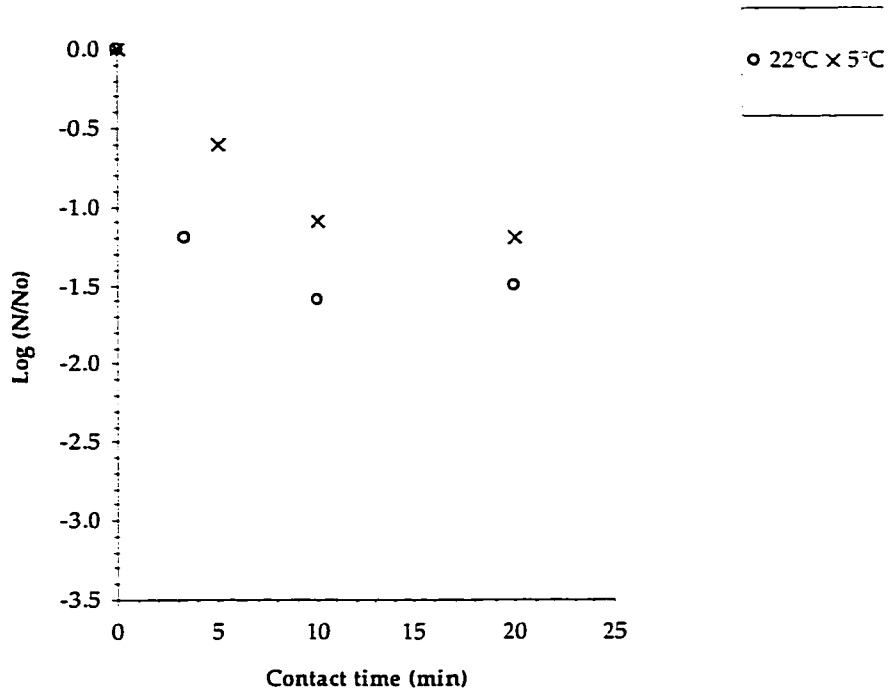


Figure 4.2 Inactivation of *B. cereus* spores by average ozone residual concentration of approximately 0.35 mg/L at pH 6 and 22°C and 5°C

4.2 KINETIC MODEL PARAMETER ESTIMATION

The Incomplete gamma Hom (IgH) model was developed using data obtained from the 13 experimental disinfection trials in 22°C water and the 10 experimental disinfection trials in 5°C water. The maximum likelihood method of model parameter estimation was used. The Solver® routine in Microsoft Excel® was used to find the maximum likelihood value. The model parameters were estimated using no fewer than 10 trials. Earlier experiments at 22°C were done without allowing for the acclimatization period of the spores. Since the spores were kept in the 4°C refrigerator they were both physiologically and morphologically adapted to cold temperature. In the later experiments when the spores were allowed to acclimatize to 22°C water there was a noticeable increase in the log inactivation. Therefore only those experiments in which the spores were allowed to acclimatize to the 22°C and 5°C water for more than 30 minutes are shown in Table 4.1 and were used to estimate the parameters of the model. The parameter estimates of the IgH models for the two temperatures are presented in Table 4.2. The upper and lower 95% confidence limits of each parameter estimates reported in the table was calculated using the liklihood ratio test (Seber and Wild, 1989):

$$\ln L_{1-\alpha}(\beta) \geq \ln L_{max}(\overline{\beta}) - \frac{1}{2}\chi^2_{p,\alpha} \qquad (4.1)$$

where L(β) is the ln liklihood and $L_{max}(\beta)$ is its supremum. First the L(β) value was calculated. Then the Goalseek® routine in Microsoft Excel® was used to

Table 4.2 Incomplete gamma Hom model parameter estimates and confidence limits

	22°C	5°C
Number of trials	13	10
pH	6	6
k ±95% k limits	1.67 1.57 to 1.76	0.88 0.83 to 0.92
m ±95% m limits	0.16 0.13 to 0.18	0.33 0.32 to 0.35
n ±95% n limits	0.55 0.42 to 0.70	0.64 0.53 to 0.75
σ	0.17	0.12
Model constraints: C = concentration (mg/L) T = contact time (min)	 $0.40 \leq C \leq 1.55$ $1 \leq T \leq 20$	 $0.35 \leq C \leq 1.71$ $5 \leq T \leq 20$

compute the limits by varying one parameter at a time while keeping the other two parameters constant at their optimal values.

Series of analyses were performed to test the validity of the model. The predicted log removal of the spores estimated by the IgH model matched closely with the actual observed log inactivation of the spores (see Figures 4.3 and 4.4). Regression residuals (obtained by subtracting the observed log percent survival from predicted log percent survival) were plotted against all variables including observed and predicted log percent survival, contact time and initial ozone residual. A typical residual plot is represented by Figure A.1. All the other plots were similar in appearance. The plots suggest that regression residuals are randomly distributed, independent of all the variables, have an approximate zero mean and a variance that is constant. To test for the normality of the regression residuals normal-scores plot outlined in Miller *et al.* (1990) was made (see Figures A.2 and A.3). The plot generally resulted in a straight line although only 13 and 10 data points were used for 22°C and 5°C respectively. Since all the assumptions of normal distribution of regression residuals are validated the model is considered adequate.

5. DISCUSSION

5.1 INACTIVATION EXPERIMENTS

During the experimental period random observation of the stock spore sample under the microscope was performed and vegetative cells were found.

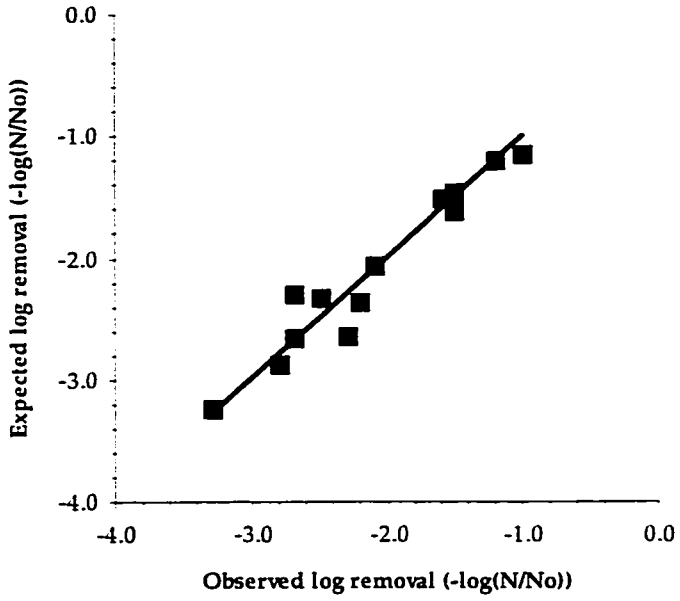


Figure 4.3 Observed *B. cereus* spore inactivation versus the *B. cereus* spore inactivation predicted by the Incomplete gamma Hom model for 22°C at pH 6

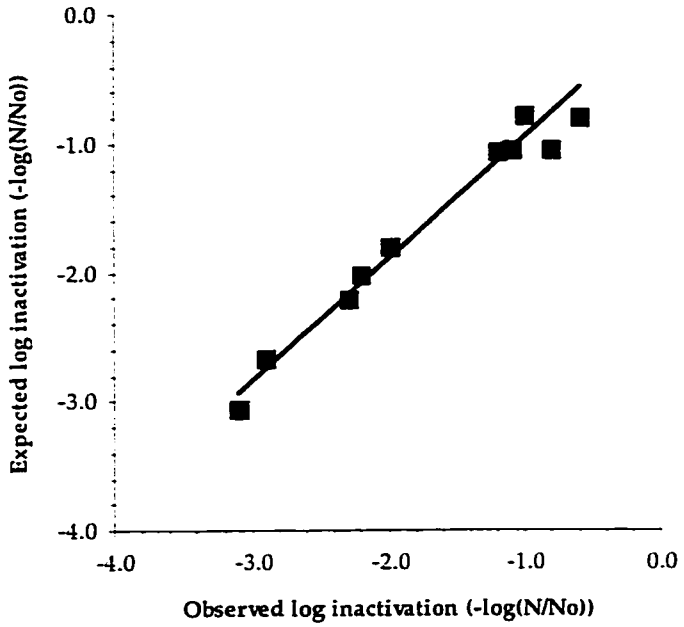


Figure 4.4 Observed *B. cereus* spore inactivation versus the *B. cereus* inactivation predicted by the Incomplete gamma Hom model for 5°C at pH 6

Vegetative cells of *B. cereus* have been reported to be 10 to 15 times more sensitive to ozone than the spores (Broadwater *et al.*, 1973). However, the percentage of the vegetative cells to the spores was very small (less than 3%). Therefore, the presence of Bacillus vegetative cells was not taken into consideration in this study. It is difficult, if not impossible to maintain pure spore suspension. After heat treatment of spores during preparation, some of the spores may have germinated. This phenomenon, referred to as heat activation, has been observed for spores of *Bacillus stearothermophilus* (Shull and Ernst, 1966).

Majority of the *B. cereus* spores were rapidly inactivated within 5 minutes but with increase in contact time the change in observed kill gradually decreased. For example, the difference in observed kill for contact time of 1 minute and 5 minute (difference of 4 minutes) was 0.6 logs for average ozone residual of 1.34 mg/L. However, the difference in observed kill for contact times of 5 and 20 minutes (difference of 15 minutes) was also 0.6 logs (see Figure 4.1). This kind of decrease in kill with respect to time has been previously reported as "retardant die-off" kinetics (Venczel *et al.*, 1996) and was observed as early as Chick's disinfection experiments with *Bacillus paratyphosus* in 1908 using phenol. Inactivation of six different enteroviruses by ozone has also shown the same "retardant die-off" kinetics as that observed in the present study (Roy *et al.*, 1982). Hom (1972) reported that variation of resistance within a population of

organisms might be an interfering factor leading to the decrease in kill with respect to time.

In the present study, when 1.34 mg/L of average ozone residual was used, a contact time of only 1 minute was necessary to inactivate initial 99% of the spores in 22°C water. A contact time of 20 minutes was necessary to inactivate 99.9% of the spores in the same temperature water. This may be indicative of a uniform *Bacillus* spore population that are sensitive to high ozone residual concentration with a small group (at most 10%) of the spore population that are extremely resistant to ozone. This trend was also observed for cold temperature experiments. When average ozone residual of approximately 1.55 mg/L was used 2 log inactivation was obtained at contact time of 5 minutes while a 3 log inactivation was obtained at contact time of 20 minutes.

The reason for the differences in the resistance of spores to ozone is not known. However, some of the possible reasons could be the thickness and quality of the spore coat or the number of the spore coat layers. Spore coat has been known to play an important role against anti-microbial agents such as lysozyme, hydrogen peroxide and chlorine (Gould and Hitchins, 1963; King and Gould, 1969; Wyatt and Waites, 1975). The resistance of spore coats to anti-microbial agents have been explained in part by the large amount of cystine disulfide bond rich proteins it contains (Vinter, 1961). Kulikovsky *et al.* (1975) have found that the spores with mutated or defected spore coats were significantly more sensitive to treatments of hypochlorite or alkali. Electron

microscopy study showed that change in permeability to the spore coat was the first major change occurring at the initial contact period with chlorine or alkali. The gradual dissolution of the spore coat with the progression of the contact period led to the destruction of the spore (Kulikovsky *et al.*, 1975). Similar kinetic studies using ozone and bacterial endospores could not be found.

Both the rates of decomposition of ozone and the reaction of ozone with substrates are greater at higher temperatures. It appears, from the present study, that generally the inactivation of spores by ozone increases with increase in temperature. For contact times of 5, 10, and 20 minutes and average ozone residual of 0.35 mg/L there was a consistent difference of 0.5 logs in observed kill (see Figure 4.2).

Miltner *et al.* (1997) carried out similar ozone inactivation experiments using bacterial spores. Some results of their investigations are summarized and compared with the present study in Table 5.1. The chlorine inactivation study using spores is also included in the table to compare the inactivation efficiency of chlorine and ozone. The water used in Miltner *et al.*'s experiment was sand filtered Ohio River water. A pilot-scale ozone contactor (single-stage, flow-through, counter-current, glass contactor) was used. The spores used in the study were indigenous but to which area was not reported in the paper. The spores were heat-treated and membrane filtered. Each colony growing on the agar was counted as one viable spore. The method used in the study by Rice *et al.*, (1996) was referenced. The range of ozone residuals and contact time

Table 5.1 Summary of chemical inactivation of bacterial spores using conventional linear Ct products

Species	Disinfectant Protocol	Water Type	Oxidant Residual (mg/L)*	Contact Time (min)	Temp. (°C)	Conventional Linear Ct for ≥ 99 Percent Inactivation (mg•min/L)	Reference
B. cereus spores	Batch reactor, Batch ozone	0.05 M Phosphate buffer (pH 6)	0.40-1.55 ozone	1-20	22	7	This study
B. cereus spores	Batch reactor, Batch ozone	0.05 M Phosphate buffer (pH 6)	0.35-1.71 ozone	5-20	5	10	This study
Various indigenous spores	Continuous flow reactor, continuous gas	Sand filtered Ohio river water (pH 8)	0.14-3 ozone	7.4	24	19	Miltner *et al.* 1997
Indigenous aerobic spores	Batch reactor, batch sodium hypochlorite	9 parts 0.05 M buffer: 1 part river water (pH 6.9)	1.60 ± 0.2 free chlorine, 1.75 ± 0.2 total chlorine	1-250	23	114	Rice *et al.* 1996

* This study defined oxidant residual (C) as the average of the initial and the final ozone residual.

Miltner *et al.* defined C as the depth-averaged dissolved ozone residual.

Rice *et al.* defined C as the mean of the chlorine residuals measured throughout the course of the experiments.

reported in their study were 0.14 to 3 mg/L and 7.4 minutes respectively. At these experimental set-up they have found that simple Ct values (calculated directly by multiplying ozone residual and contact time for observed log inactivation) required for 1 and 2 log inactivation of naturally occurring aerobic endospores were approximately 6.2 and 18.5 mg•min/L respectively. The C in the calculation of the Ct was based on the depth-averaged dissolved ozone residuals measured from various sampling ports of the contactor. The approximate Ct values calculated from the present study are 0.5 and 7.2 mg•min/L for 1 and 2 log inactivation respectively. Comparison of the present study with that of Miltner *et al.* (1997) using simple Ct values suggest that indigenous spores are more resistant to ozone than pure culture laboratory spores. The apparent difference in resistance may also be, in part, due to the different water used. Ohio River water has a greater ozone demand and faster decay than the water used in the present study.

Rice *et al.* (1996) carried out similar spore inactivation experiments using chlorine. They also used indigenous aerobic spores found in the river water. Chlorine in the form of dilute sodium hypochlorite was added to batch-type reactor containing 1 part local river water and 9 part 0.05 M chlorine demand free phosphate buffer and approximately 15,000 colony forming units/100 mL. For all experiments the mean free chlorine residual applied was 1.60 ± 0.2 mg/L. The contact time range reported was 1 to 250 minutes. Simple Ct values reported for 2 and 3 logs were 114 mg•min/L and 315 mg•min/L respectively. Upon

comparing the Ct values with either the present study or Miltner *et al.*'s study, it is clear that ozone is much more effective at killing aerobic spores than chlorine. Their survival plot of indigenous spores in diluted river water was also indicative of the retardant die-off kinetics.

Interestingly, Rice *et al.* reported that, when compared to chlorine disinfection experiment performed by Williams and Russell (1991), pure culture spores have greater resistance to ozone than indigenous spores. It should be pointed out that a major difference between these two studies is the level of reported chlorine residual concentrations. Williams and Russell (1991) used high chlorine concentrations of up to 100 mg/L of chlorine residuals while Rice *et al.* (1997) used only 1.60 mg/L of chlorine residual throughout their experiment. When comparing Ct values, disinfectant residual concentrations and contact times should be specified. For example, Williams and Russell's study reported a Ct value of 500 mg•min/L for 3 logs using average chlorine concentration of 100 mg/L but when they used average chlorine concentration of 50 mg/L, Ct value calculated for 0.7 log inactivation was 750 mg•min/L. Another noticeable difference between Rice *et al.*'s study and Williams and Russell's study is the methodology each group employed. Rice *et al.* added spores and chemicals to a batch reactor containing a solution of 1 part river water and 9 part buffered water whereas Williams and Russell added spores to a solution of 20 mL volumes of nutrient broth containing the appropriate concentration of a biocide. A parallel study of pure cultured and indigenous spores using same conditions is

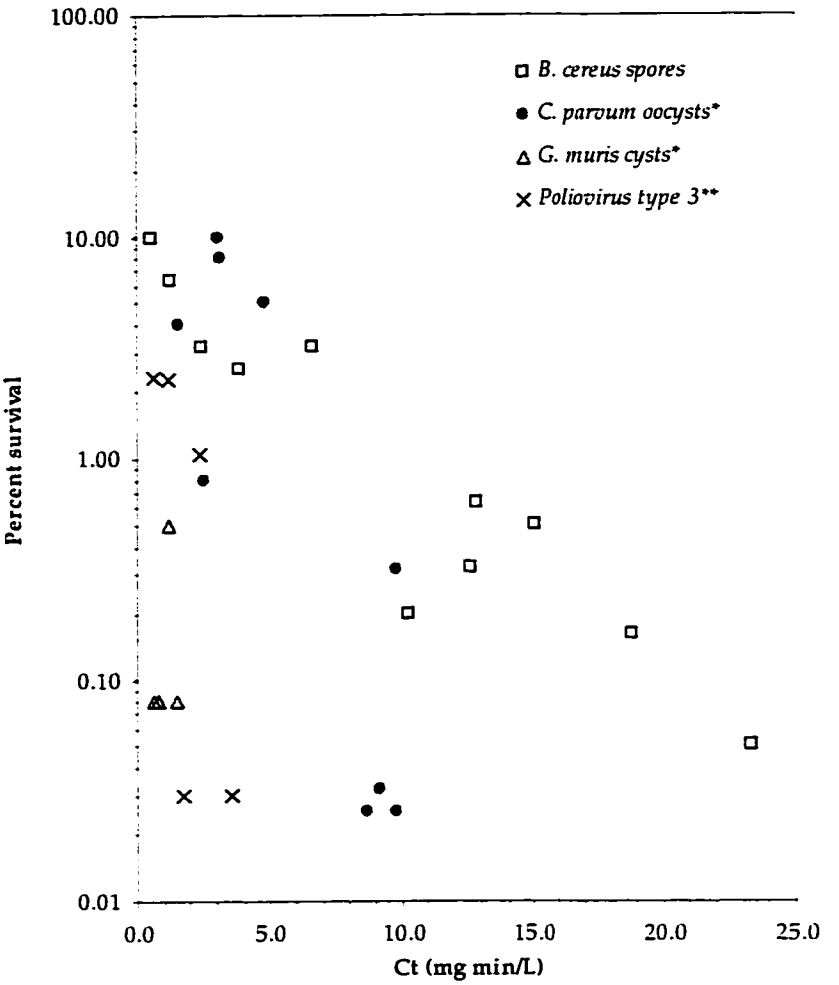
necessary to determine which spores are the more resistant ones. Hirata *et al.* (1991) carried out a parallel study of indigenous and cultured spores of *Clostridium perfringens* using chlorine as a disinfectant and reported that indigenous species are about 3 to 5 times more resistant than the cultured ones.

5.2 COMPARISON OF MICROBIAL INACTIVATION BY OZONE

Bacterial spores have been suggested as an indicator of water-borne pathogens (Hirata *et al.*, 1991; Payment and Franco, 1993; Hijnen *et al.*, 1997; Miltner *et al.*, 1997). Miltner *et al.* has performed a series of experiments at similar conditions that have allowed their group to compare the ozone inactivation of bacterial endospores with *C. parvum* oocysts, *G. muris* cysts, and Poliovirus 1. The authors of the present study have also performed collective ozone inactivation experiments of the same organisms. The results are compiled in Figure 5.1. The figure is a replica of Figure 1 in Miltner *et al.* (1997). The main differences between the two experiments are the kind of water used and the type of reactor employed. Figure 5.1 is remarkably similar to Figure 1 in Miltner *et al.* (1997). It is clear from Figure 5.1 that bacterial spores rank higher in resistance to ozone inactivation than either *G. muris* cysts or Polioviruses. Figure 5.1 show that at some point spores rank higher than even the *C. parvum* oocysts.

5.3 INACTIVATION KINETICS

In order to obtain a closer understanding of the ozone inactivation of the spores the experimental results were used to develop a kinetic model. The



Notes: * Finch *et al.* (1994)

** Finch and Fairbairn (1991)

Figure 5.1 Comparison of the microbial inactivation by ozonation in 22°C, neutral pH ozone demand free phosphate buffered water

parameter estimates (see Table 4.2) calculated show that both *m* and *n* values are significantly less than one. This suggests that reporting of simple Ct (average disinfectant residual multiplied by defined contact time) values for the purpose of describing the relative resistance of bacterial spores to disinfectants may not be reliable. Table 5.2 lists Ct ranges calculated from series of C and t generated by the IgH model for *B. cereus* spores. The model predicts that for 2 log inactivation of *B. cereus* spores a Ct value of anywhere between 0.60 and 22 may be used. Kinetic analysis of disinfection data indicate that Ct values do not remain constant for a given level of inactivation but are subject to variations depending on the disinfectant residual and contact time used.

For *B. cereus* models *m* values were significantly lower than the *n* values for both 22°C and 5°C data. This was especially noticeable with the 22°C data. The value of the *m* nearly doubled when the water temperature was changed from 22°C to 5°C while the value of the *n* stayed approximately the same. This suggests that contact time may have a greater influence on the inactivation of spores at low temperatures than at high temperatures. The low value of *m* relative to *n* is characterized by the tailing-off effect of the curves in Figure 4.1. At 5°C, where the difference in *m* and *n* is less than the difference at 22°C, the tailing-off effect is relatively less severe.

The kinetic study of *Cryptosporidium parvum* oocyst inactivation by ozone has been done by Gyürék (1997). Both the methodology used in this study and ozone residual concentration and contact time range parallels that which was

Table 5.2 Ct ranges calculated using data generated by Incomplete gamma Hom model for *B. cereus* spores and *C. parvum* oocysts

-log (N/No)	22°C		5°C	
	B. cereus	*C. parvum**	*B. cereus*	*C. parvum**
1	0.1 - 0.8	1.3 - 1.6	0.8 - 4.0	3.0 - 4.0
2	0.6 - 14.8	3.9 - 5.0	6.6 - 28	7.4 - 9.8
3	7.6 - 37.2	7.2 - 9.3	26 - 55	12.6 - 16.6

Notes: *Values were originally reported in (Gyürék, 1997).

*The low temperature value for *C. parvum* is at 7°C.

Table 5.3 Comparison of the results from ozone inactivation study of *Cryptosporidium parvum* oocysts and *Bacillus cereus* oocysts

Parameters	*C. parvum*	*B. cereus*
Disinfectant	Ozone	Ozone
pH	6	6
Temperature (°C)	22, 7	22, 5
Model constraints		
Concentration (mg/L)	0.6 to 2.9	0.35 to 1.71
Contact time (min)	2.9 to 14.9	1 to 20
Room temperature parameters		
k	0.82	1.67
Confidence limits*	0.66 to 0.99	1.57 to 1.76
m	0.65	0.16
Confidence limits	0.53 to 0.74	0.13 to 0.18
n	0.42	0.55
Confidence limits	less than 0 to 0.98	0.42 to 0.70
Cold temperature parameters		
k	0.48	0.88
Confidence limits	0.41 to 0.55	0.83 to 0.92
m	0.72	0.33
Confidence limits	0.64 to 0.79	0.32 to 0.35
n	0.79	0.64
Confidence limits	0.48 to 1.06	0.53 to 0.75
Reference	(Gyürék, 1997)	This study

Note: *Confidence limits shown for *C, parvum* oocysts and *B. cereus* spores are 95% confidence limits.

used by Gyürék's study and therefore the two results can be directly compared. Although the parameters, *m* and *n*, for the oocysts are less than one their values are similar to each other. The reason for this may be that, unlike the spores, oocysts have either a uniformly resistant population or have a heterogeneous population possessing an approximately normal distribution of ozone resistancy. Having similar *m* and *n* values also means that concentration and contact time will have near equal influence on the level of inactivation. The direct result of this is a considerably less tailing off effect of the survival curves and considerably narrower Ct ranges. The comparison of the parameter estimates (see Table 5.3) show that *n* values are similar but *m* values are different. At room temperature the value of *m* for *B. cereus* model is as much as 4 times the value of *m* for *C. parvum* model, and at 5°C the differences is as much as 2 times. Because of this difference in *m* the inactivation rate constant *k* could not be directly compared.

The Ct ranges reported for *C. parvum* using data generated from IgH models is tabulated in Table 5.2 for comparison with that of *B. cereus* spores. At 22°C, higher Ct value was necessary for 1 log inactivation of *C. parvum* oocysts than for *B. cereus* spores (1.3 to 1.6 mg•min/L verses 0.1 to 0.9 mg•min/L respectively). The Ct range for 2 log inactivation of *B. cereus* spores was considerably wide. Therefor, depending on the concentration and contact time used, it can be said either higher or lower Ct value is required for 2 log inactivation of *C. parvum* oocysts than for *B. cereus* spores (3.9 to 5 mg•min/L compared to 0.6 to 22 mg•min/L respectively). However, it generally required

greater Ct values to achieve greater than 3 log inactivation of *B. cereus* spores than *C. parvum* oocysts (8.8 to 34 mg•min/L verses 7.2 to 9.3 mg•min/L respectively). The trend was similar when cold temperature experiments were compared. Miltner *et al.* (1997), using ozone, also found that for up to 1 log inactivation less Ct value was required for indigenous bacterial spores than for *C. parvum* oocysts. But for greater than 1 log inactivation they found that considerably higher Ct value was required for indigenous spores than for *C. parvum* oocysts. The results of this study indicate that caution should be taken when making comparisons using Ct values.

The comparison of log percent removal versus contact time of *C. parvum* oocysts and *B. cereus* spores using ozone residual of 0.5 mg/L and is illustrated in Figure 5.2. The figure shows that there is an explicit "tailing-off" effect for *B. cereus* inactivation while inactivation of *C. parvum* is more gradual. Depending on the contact time *B. cereus* spores may be subjected to higher levels of inactivation or considerably lower levels of inactivation than *C. parvum* oocysts. While the overall effect of contact time regarding ozone disinfection of spores is minimal the overall effect of contact time on *Cryptosporidium* is equally, if not more important, than ozone residual concentration. Therefore, *B. cereus* spores may not be appropriate indicators of ozone inactivation of *C. parvum* oocysts. Figure 5.2 clearly shows that while it is not practical to design a new disinfection facility using kinetic models of spore inactivation.

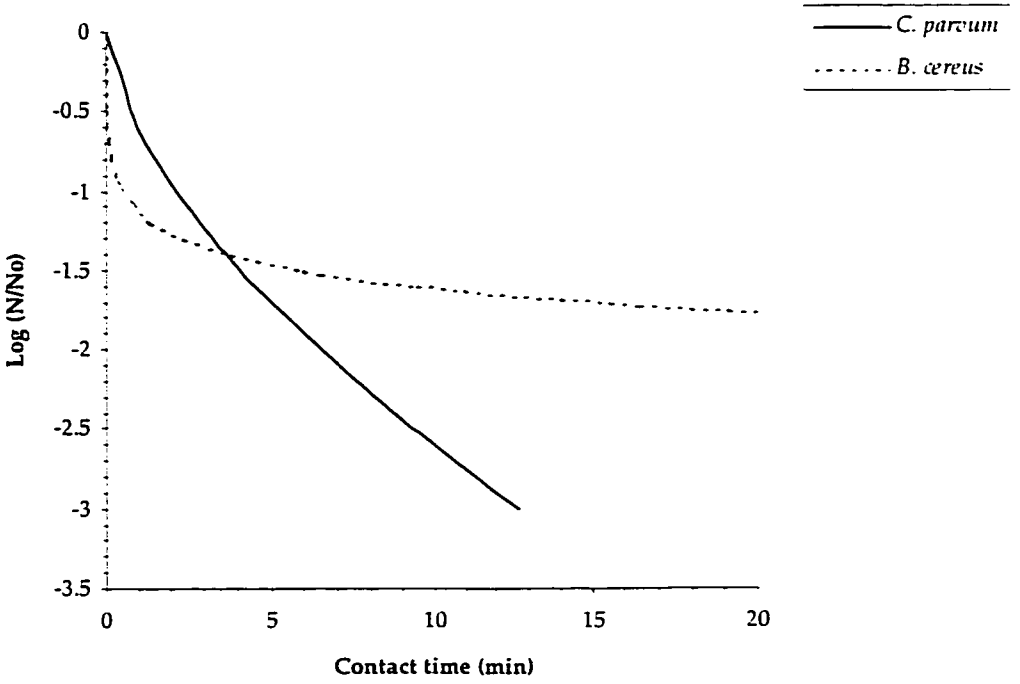


Figure 5.2 Survival plot of *B. cereus* spores and *C. parvum* oocysts using 0.5 mg/L of ozone residual at pH 6, at 22°C, and first order ozone decay rate constant of 0.03 min^{-1}

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The list of conclusions drawn from this study is:

- Generally, ozone concentration has a greater impact on the inactivation of *B. cereus* spores than contact time;
- the ozone inactivation of the *B. cereus* spores are characterized by "retardant die-off" kinetics;
- less contact time and/or ozone residual is required for initial 0.5 and 1 log removal at 22°C than at 5°C, but for 3 log removal same contact time and/or ozone residual is required at both temperatures;
- up to 1 log removal less contact time and/or ozone residual is required for *C. parvum* oocysts than *B. cereus* spores, but it is much more difficult to achieve greater than 1 log removal of *B. cereus* spores than *C. parvum* oocysts;
- *B. cereus* spores are significantly more resistant to ozone than Poliovirus and *G. muris* cysts; and
- *B. cereus* spores are poor indicators of *C. parvum* oocyst inactivation using ozone.

6.2 RECOMMENDATIONS

The following recommendations are proposed.

1. An in-depth disinfection study using indigenous spores with a diverse population of aerobic spores should be performed. This may provide a better comparison with the *Cryptosporidium* inactivations.
2. A parallel study of pure cultured spores and indigenous spores using same experimental conditions is recommended in order to determine which population is more resistant to ozone.
3. The predictability of ozone spore disinfection in natural waters representative of water treatment plant conditions using the model developed in this study should be considered for investigation.
4. More inactivation data should be collected at both of the temperatures in order to confirm that the value of the Incomplete gamma Hom model parameter, m, is indeed different for both of the temperatures.
5. Experiments at 1°C water should be investigated to examine whether the trend in inactivation from 22°C to 5°C is continuous to 1°C.

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APPENDIX A – REGRESSION RESIDUALS PLOT

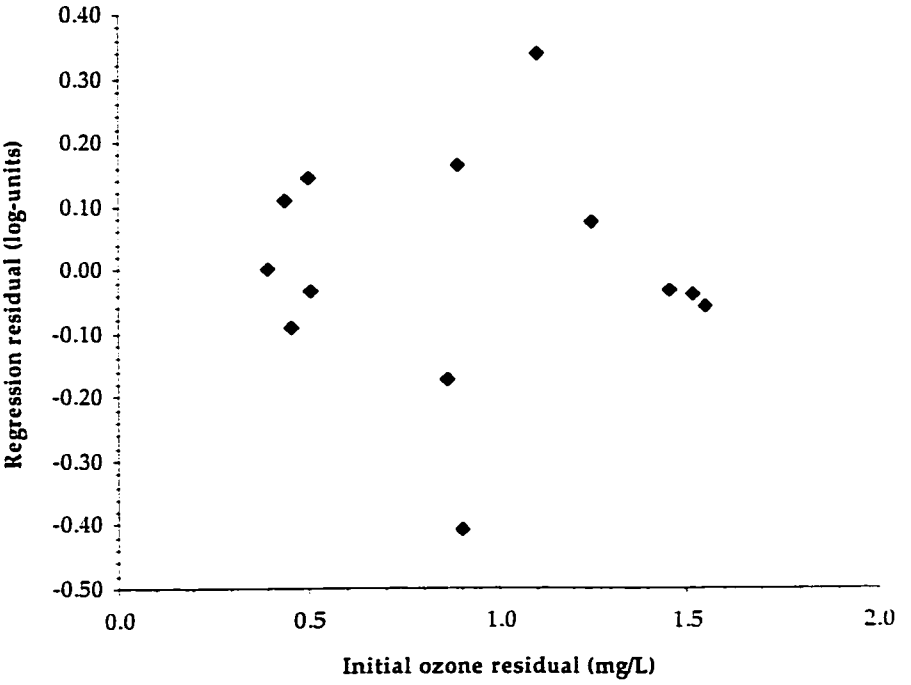


Figure A.1 Regression residuals plotted against the initial ozone residual

APPENDIX B - NORMAL SCORES PLOTS

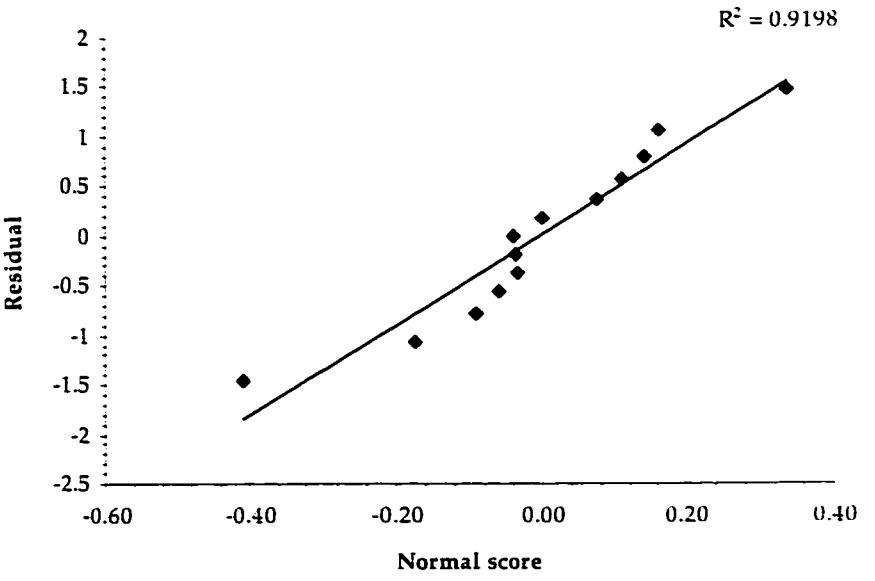


Figure A.2 Normal-scores plot of the 22°C inactivation data residuals

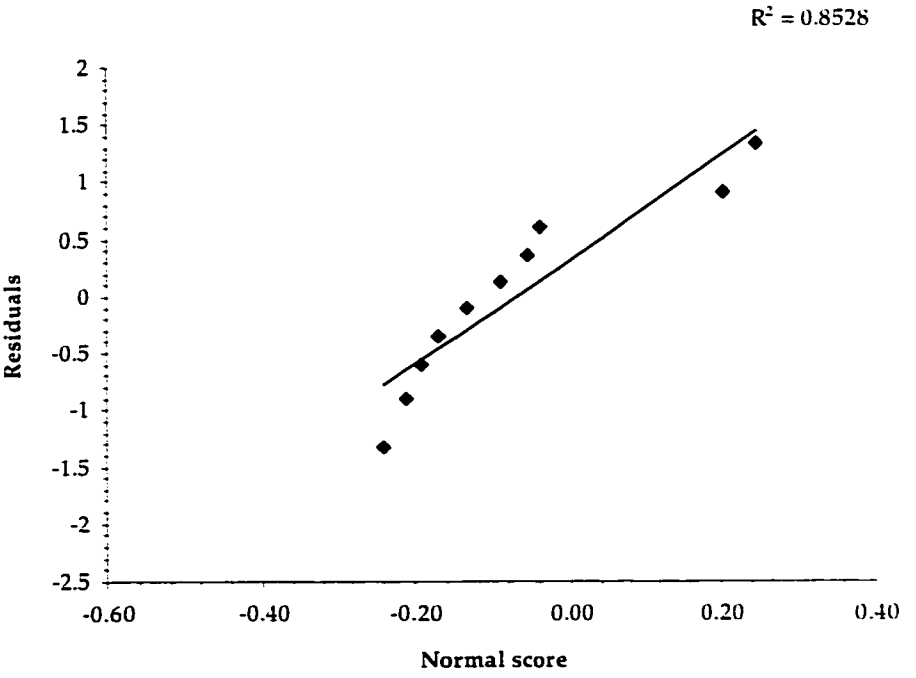
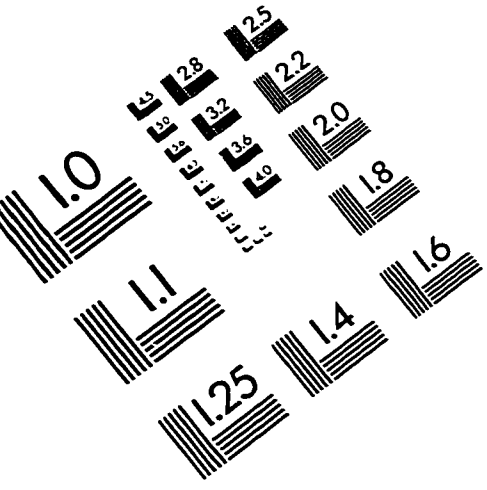
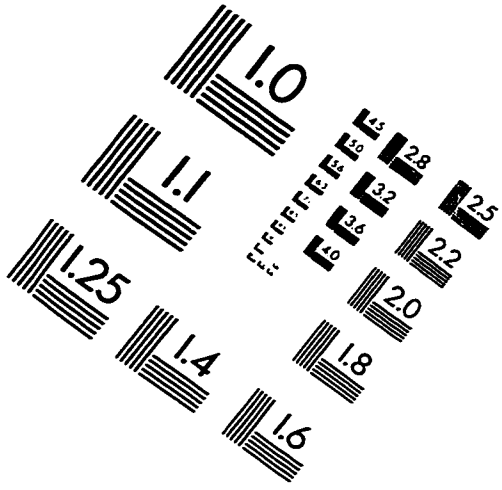
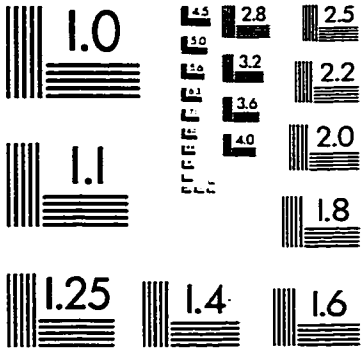


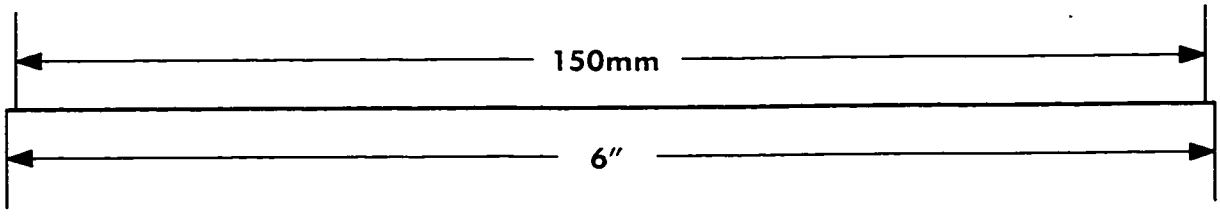
Figure A.3 Normal-scores plot of the 5°C inactivation data residuals

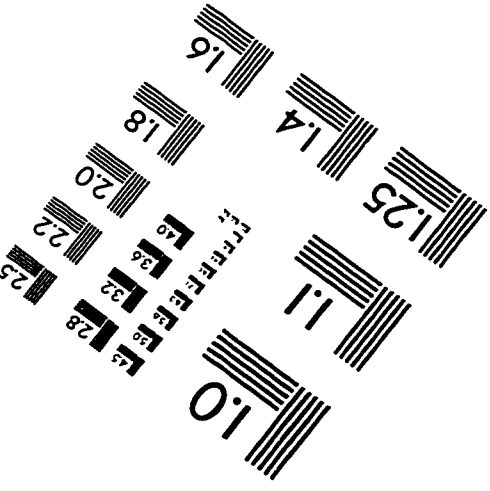
IMAGE EVALUATION
TEST TARGET (QA-3)

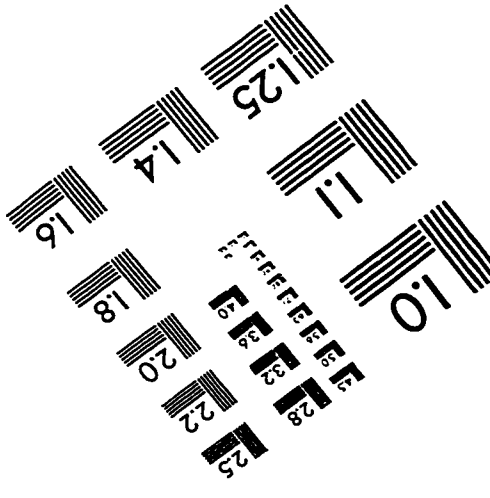












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