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Application of gaseous disinfectants ozone and chlorine dioxide for inactivation of Bacillus subtilis spores

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Application of Gaseous Disinfectants Ozone and Chlorine Dioxide
for Inactivation of Bacillus Subtilis Spores

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy in
Engineering Sciences (Applied Mechanics)

by
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2006
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is acceptable in quality and form for publication on
microfilm:

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University of California, San Diego

2006
dedicated to

loving memory of my father

Bilal Aydogan

1940-2002
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LIST OF SYMBOLS AND ABBREVIATIONS

B. subtilis sp. = Bacillus subtilis species

c.f.u. = colony forming units

ClO$_2$ = Chlorine dioxide

CSTR = Complete stirred mixed reactor

CT = Concentration × time multiplication

DPA = Dipicolinic acid

HVAC = Heating, ventilation, and air-conditioning

NB = Nutrient broth

O$_3$ = Ozone

ppb = Part per billion

ppm = Part per million

RH = Relative humidity

RT = Retention time

S1 = Stock spore solution – 1

S2 = Stock spore solution – 2

SWTR = Surface water treatment rule

TSA = Tryptic soy agar

TSB = Tryptic soy broth

VOCs = Volatile organic carbons

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ABSTRACT OF THE DISSERTATION

Application of Gaseous Disinfectants Ozone and Chlorine Dioxide for Inactivation of Bacillus Subtilis Spores

by

Ahmet Aydogan

Doctor of Philosophy in Engineering Sciences (Applied Mechanics)
University of California, San Diego, 2006
San Diego State University, 2006

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A terrorist attack involving chemical and/or biological warfare agents is a growing possibility. Since anthrax is considered as an immediate public-health threat that can be created by a warfare agent, it is imperative to investigate the potential remediation technologies effective against this threat. In this study, the effectiveness of two gaseous disinfectants, ozone and chlorine dioxide, to inactivate \textit{B.subtilis} spores – as surrogate to \textit{B.anthracis} that can cause the infectious anthrax disease – was investigated. The inactivation rates and mechanisms of the spores by these disinfectants were investigated in both the gaseous and aqueous phases under various experimental conditions.
In the gaseous phase, chlorine dioxide was found to be at least ten times more effective than ozone for inactivation of the spores under the similar conditions. The rate of inactivation by both disinfectants increased with increasing gas concentration, contact time and relative humidity (RH). Inactivation was found impractical at humidity levels below 70%. The concentration×time, or the CT concept, was valid only under limited conditions. A diffusion limited disinfection model was proposed to mechanistically explain the deviations from the linear CT rule. The experiments with different surface materials showed that the type of surface on which the spores rest may affect the inactivation rate significantly.

In the aqueous phase, the CT concept was applicable to the inactivation rate data for both disinfectants. Ozone was shown to be much more effective than chlorine dioxide in inactivating spores in the aqueous phase.

The presence of the main growth nutrients was found to be essential for the growth and the resistance of the spores. The presence of several cations, especially magnesium and manganese, in the growth medium was required to create spores with high resistance against the gaseous disinfectants.

In conclusion, this research provides the groundwork for application of ozone and chlorine dioxide for remediation of an enclosed space that is subject to contamination by biological agents. In addition, it offers valuable kinetic and mechanistic information on the inactivation patterns of *B.subtilis* spores by ozone and chlorine dioxide in both the gaseous and the aqueous phases.
CHAPTER 1

INTRODUCTION AND THEORETICAL BACKGROUND

1.1 INTRODUCTION

Tools and strategies are being developed in response to a possible terrorist attack involving chemical and/or biological warfare agents. These include early detection sensors, crisis management planning, forensics, and decontamination strategies. Anthrax is considered as the most current and immediate public health threat that can be created by a warfare agent. Anthrax was pointed out as one of the most serious threats in a consensus paper published in 1999 (1), well before the 2001 incidents. Anthrax is caused by *Bacillus anthracis* spore, a microorganism that adapts well to extreme environmental conditions. When properly prepared, the anthrax spores can be aerosolized and become available in lethal dosages for inhalation or body contact. The spores may be introduced into the ambient air from opened contaminated packages or envelopes, as well as through the ventilation systems of buildings.

Methods to kill bacteria and other infectious agents in the production of pharmaceuticals, foodstuffs, and in microbiology laboratories are well understood.
However, decontamination of microbiological agents used in warfare or terrorism, especially in a domestic environment, presents entirely different challenges.

To be useful, a decontaminant must be reactive, yet non-corrosive, safe to use on sensitive equipment like computers, effective on a broad spectrum of chemical and biological agents, and environmentally safe. There are two general categories of methods for decontaminating biological and/or chemical agents – reactive gases and liquid solutions and foams. Gases have a presumed advantage over foams and liquids in that they are believed to be able to reach into every nook and cranny of a room. Entire rooms may be decontaminated by filling them with high concentrations of a chemically reactive gas, such as formaldehyde, methyl bromide, ethylene dioxide, propylene oxide, chlorine dioxide or ozone gas (2).

Ozone and chlorine dioxide gases are expected to be the most appropriate and effective disinfecting agents for decontamination of air and the surfaces in rooms, dwellings, offices, buildings, etc. that may have been exposed to anthrax spores.

1.2 BACKGROUND

In this section, the theoretical background for ozone and chlorine dioxide gases, bacterial spores’ structures and inactivation curves specifics, disinfection kinetics, and possible reactions between the carrier material and the gases are presented.
1.2.1 Ozone

Ozone (O₃) is a chemical that functions as both a strong oxidant and a disinfectant. Ozone has been used commercially for almost 100 years to kill many types of bacteria, viruses, spores, molds and fungi, and oxidize many types of undesirable organic and inorganic contaminants in potable waters and wastewaters. It is the choice disinfectant of many drinking water facilities in the U.S. and throughout Europe and Asia because of its ability to inactivate resistant spores and cysts, such as Cryptosporidium and Giardia. Recently, the Food and Drug Administration (FDA) has accepted ozone as a disinfectant of food contaminants. Ozone received the Generally Regarded as Safe (GRAS) status in 1997, and became an unrestricted food additive in July of 2001. The superiority of ozone over other water disinfectants has been clearly established in the literature. In 1991, the USEPA confirmed that ozone is the most effective primary disinfectant available for drinking water (3).

Ozone is generated in gas form from pure oxygen or air. Because of its instability (ozone reverts to oxygen), it must be generated and applied onsite. It can be used directly in the gas phase to inactivate air-borne microorganisms, or dissolved in water to inactivate microorganisms in water. Earlier studies showed the effectiveness of ozone in inactivating spore-forming bacteria in water including Bacillus species. These species include the non-pathogens Bacillus subtilis and Bacillus globigii spores, which belong to the same genus as Bacillus anthracis that cause anthrax. The effectiveness of ozone on B. anthracis as well as on other potential biowarfare agents,
including toxin of *Clostridium botulinum* and influenza virus in ozonated water was demonstrated through a study conducted for the Chemical Corps of Fort Detrick, Maryland as early as 1956. This study showed that ozone was equally effective on *B. anthracis* and *B. subtilis* (4).

Prior research showed that ozone is also effective on bacterial spores in gas phase applications. Ishizaki *et al.* (5) examined the sporidical activity of ozone on different *Bacillus* spores for different ozone concentrations between 250 and 1500 ppm. The difference between the inactivation rates of different strains was relatively small at high relative humidity of 80 to 90%. About 5-log reduction was obtained in less than two hours. Currier *et al.* (6) presented experimental results on *B. globigii* spores at an ozone concentration of 9000 ppm. Application of ozone was very effective against *Bacillus* spores at this high concentration by leaving undetectable numbers (more than 4-log reduction) in less than 1 hour. Both Ishizaki *et al.* and Currier *et al.* observed ozone to be less effective at humidity levels below 60%. There are also a few studies that reported the use of gaseous ozone for decontamination of bioclean rooms in hospitals (7, 8), dental equipment sterilization (9), and laboratory facilities sterilization (10). These studies demonstrated the feasibility of spore inactivation by gaseous ozone. However, additional information is needed on the rate of inactivation of the spores at various ozone concentrations, humidity, and the type of material surface that the spores rest, as well as the physical effect of ozone on the material.
1.2.2 Chlorine Dioxide

Chlorine dioxide (ClO₂) was first produced from the reaction of potassium chlorate and hydrochloric acid by Sir Humphrey Davy in 1811 (11). However, its widespread use did not occur until the industrial scale preparation of sodium chlorite, from which chlorine dioxide may more readily be generated (12).

Chlorine dioxide is being used increasingly to control microbiological growth in a number of different industries including the dairy, the beverage, the pulp and paper, the fruit and vegetable processing, the poultry, and the beef processing industries, as well as miscellaneous food processing applications (13, 14). Despite its early investigation as an oxidant and disinfectant (13), ascendancy of chlorine dioxide in both water and wastewater treatment has been slow.

Chlorine dioxide is generated on an as needed basis by a controlled chemical reaction. Generation of the chemical is generally the reaction of sodium chlorite with either an acid or chlorine. In drinking water disinfection, the target organisms of concern are disease causing viruses, actively metabolizing vegetative bacteria, and pathogenic protozoa such as *Giardia* and *Cryptosporidium*. Bacterial spores have not been the target organisms because they have not generally been regarded as important waterborne pathogens. Very recently, investigators have started to assess the removal of spores through water treatment processes including disinfection since their resistance approximates the most resistant protozoa of concern (15-20).
The use of chlorine dioxide as a disinfectant/sanitizer in gas phase has been studied over the past 20 years. In 1988, the EPA registered chlorine dioxide gas as a sterilant. In the medical sterilization field, the efficacy of a gaseous chlorine dioxide process was examined using the spores of *Bacillus subtilis* as a test organism (21). The results of this study indicated that performance of chlorine dioxide varied with temperature and humidity. At a relative humidity of 80% and a temperature of 30°C, the time required for one log inactivation (90%) was 4.4 minutes at a gas phase concentration of 30 mg/L. Yet no published information has been encountered in the refereed scientific literature concerning either the use of gaseous chlorine dioxide as a decontaminating agent for large buildings or spaces, or on the sensitivity of biological threat agents to either gas phase or liquid phase chlorine dioxide.

In October 2001, several places in the East Coast of the US fall victim to the largest bioterrorist attack in the history of the US. These attacks caused the closures of several facilities: Hart Senate Building and other Capitol Hill offices and one post-office in Washington, D.C., one post-office facility in New Jersey, and one publishing facility in Florida. The Hart Senate Building was decontaminated using chlorine dioxide gas and liquid foams. The procedure followed was as follows (22): The building was sealed to prevent chlorine dioxide gas leakage to the surrounding areas, and windows were blacked out to minimize light exposure. In preparation for fumigation, three thousand test strips were placed in the office where the contaminated letter was found. The preliminary experiments showed that the humidity
should be held at 75% and the temperature should be kept above 20°C throughout the application process. Once the humidity was raised to the identified level by pumping in steam, chlorine dioxide gas was introduced into the building through the HVAC system. Initially, 500-550 ppm chlorine dioxide gas applied for 12 hours for a total of 6000 ppm-hours but test strips showed that decontamination was not complete. Later, the concentration was increased to 750 ppm for another 12 hours exposure for a total of 9000 ppm-hours. This time, the test strips showed complete decontamination. This same procedure was applied to decontaminate the other facilities but the details of neither application were published.

1.2.3 Bacterial Spores

Bacterial spores are amongst the most dormant and the most resistant living microorganisms known. They are formed by bacilli and clostridia species in response to exhaustion of a nutrient during an approximately 8-hour developmental process, called sporulation, which is controlled by a complex cascade of cellular events (23, 24). Dormant spores of these species are much more resistant than their vegetative cell counterparts to a variety of treatments including heat, UV radiation, and oxidizing agents (25-34). Spores can persist in the dormant state for very long and perhaps even geological time scales (35, 36). Nonetheless, the spore is not insensitive to its surroundings. Rather, it is continuously poised to react to the reintroduction of even minute amounts of nutrients to the environment (24). The result is the almost
immediate conversion of the spore back to actively growing cell, a process known as germination.

The spore is composed of a set of protective structures arranged in a series of concentric shells. Each component contributes in some essential way to spore’s durability. Resistance depends on three spore substructures: the core, the cortex, and the coat. Figure 1.1 shows a diagram of basic spore structure. The spores of *B. subtilis* are in ecliptic shape. The mean size of dormant spores was found to be 1.2μm long and 0.8μm wide (37).

![Figure 1.1 Diagram of basic spore structures](image)
1.2.3.1 Spore Core

The interior compartment of the spore, the core or protoplast, contains the cell’s normal cytoplasmic constituents - the DNA, ribosome and most of the spore’s enzymes. One of the main reasons for spore resistance is the low water content in the spore core (33). The core also contains dipicolinic acid (DPA) which has more than 15% of the core dry weight. For all life forms, DPA is unique to bacterial spores. DPA is accumulated late in spore formation and is excreted in the first minute of spore germination (38, 39). DPA in the spore core is in chelate form with an equimolar amount of divalent cations, predominantly calcium. This chelate is likely present in a relatively dehydrated crystalline lattice which increases the stability and the resistance of spore core (40). The spore chromosomes are saturated with small acid soluble proteins (SASP) which makes up about 20% of core proteins. They play a significant role for resistance of the spore DNA against radiation, heat and some chemical oxidants. The SASP are normally rapidly degraded during germination and provide amino-acids for the germinated spore (27, 30).

1.2.3.2 Core Membrane

The spore core is surrounded by an inner membrane which serves as the major permeability barrier restricting the passage of small molecules into the spore core (39-41). The core membrane is composed of protein and lipids in structure. It is only permeable to small uncharged molecules with molecular weight less than 150 Da
However, the rate of permeation of small uncharged molecules into the spore core is extremely slow, possibly because of the compressed state of the spore’s inner membrane (43, 44), and the condition of the membrane lipids being mainly in the immobile form (40). The lipids present in the inner membrane of dormant spores were shown to be immobile. This suggests the presence of a substantial amount of gel-phase lipid that would be expected to greatly decrease the passive permeability of this membrane (40, 45-47).

### 1.2.3.3 Spore Cortex

The core membrane is surrounded by a thick layer of specialized, loosely cross linked peptidoglycan called cortex. The cortex is about 100 nm wide in *B. subtilis* spores (41). The tight girdle formed by the cortex acts to keep the core relatively dry and the membrane in the compressed state. The cortex resembles more of outer structure of a bacterial cell rather than an intracellular structure. It is very similar to bacterial cell wall topographically and functionally as well is in its composition. Because the cortex has a structure resembling a woven fabric, small molecules, such as water, can pass through it. Despite this, the core remains dry due to the squeezing action of the cortex on the core (48). In addition, the cortex can have an osmotic pressure as high as 2 MPa (25, 38, 49) due to its electronegative peptidoglycan polymers and positively charged counter-ions (cations) composition. This may result in extra resistance against the penetration of large molecules through the cortex (25).
1.2.3.4 Spore Coat

The cortex is encased in a multilayered protein shell called the spore coat. An additional membrane set, the outer membrane, may also be present between the cortex and the coat in some spore species (50, 51). The outer membrane is similar to inner membrane in composition but with a simpler structure (50). The spore coat has critical roles in protecting the spore from a variety of toxic molecules and enzymes (50, 52, 53) as well as in facilitating the germination (51, 54, 55). The coat appears as a series of concentric layers in the electron microscope. The number and fine structure of layers differ for different species. The coat has been deeply studied only in the model organism *B. subtilis*. In *B. subtilis*, there are two major coat layers; an inner coat and an outer coat (50). The inner coat has a fine lamellar appearance and stains lightly. It is composed of several (between two and five, usually about four) layers and is about 75 nm wide. The inner coat is surrounded by the outer coat, which is often thicker than the inner coat ranging from 70 to 200 nm wide (50). The outer coat stains darkly and has a more coarsely layered appearance. In *B. subtilis*, the layers of coat were detected to have fibrous morphology (50). The fibers in two layers run at right angles to each other. The coat's morphological complexity is mirrored by the complexity of the coat's polypeptide composition: At least 40, and perhaps as many as 60, protein species are present in the coat (56). Frequently, the outer circumference of the spore appears scalloped (50). A recent study revealed that the coat surfaces are populated by a series of bumps ranging between 7 and 40 nm in diameter depending on the species (56). The degree of the porosity of spore coat is not known but the size of molecules
that can pass through the coat has been estimated to be approximately 8 to 2kDa. The studies suggest that the permeability through the spore coat should be similar to a sieving function such as decreasing pore dimensions through the layers (50). One function of the coat is to exclude large toxic enzymes, such as lysozyme (57) that degrades the peptidoglycan in cortex. Since the radius of gyration of lysozyme is about 2nm (58), it is likely that pores in the spore coat should have a radius less than 2nm at some level.

1.2.3.5 Germination

The process of the spore converting from the dormant state back to vegetative growth is called “germination”. In this phase, when spore senses a nutrient in the environment (usually a sugar or some other small molecule), it begins the process of rehydration that precedes the resumption of metabolism in the core (59, 60). The rehydration is rather fast; within 10 min of exposure to rich medium, spores can increase in size to about 1.8μm long and 1.2μm wide on average. Additionally, the ridges and bumps on the coat surfaces can disappear (56). During this initial period, the DPA present in the core is released and an enzyme present in the coat helps for degradation of the cortex. The cortex diminishes in width and the peptidoglycan layer becomes the cell wall of the outgrowing cell. Finally, the coat cracks open to liberate the revived cell (50).
1.2.3.6 Inactivation Mechanisms

The studies on spores indicate that there could be three major mechanisms of spore killing (61): (i) First one is through the DNA damage and prevention of spore from replication. The major disinfection methods that kill partly by this mechanism are UV and dry heat (33, 34). (ii) The second mechanism would be the disruption/destruction of the spore’s inner membrane permeability barrier. The destruction of the membrane may result in the release of protoplast content into environment and prevention of the regrowth. Even if the membrane is not destroyed completely, its permeability may increase allowing the entrance of external enzymes and/or oxidizing agents into the spore core. In addition, the external effects may degrade cortex resulting in the hydration of the spore core and an increase in the volume, making the spore more susceptible to further external effects. The increase in core volume should bring in an increase in the membrane area and the pore diameter, leading to higher permeability into the spore core. (iii) The third possible mechanism for spore killing is by inactivation of one or more components of the spore germination apparatus (61, 62). Some killing agent may inactivate one of the vital enzymes, located in the spore core or coat, or mechanisms required during germination or outgrowth which will result in the loss of viability for the spore. Several studies reported the initiation of the germination by inactivated spores, however without the further growth (34, 39, 61, 63-66). Some of the chemical disinfectants that initiated germination include hydrogen peroxide (63), sterilox (65), peroxynitrite anion (61), and CuCl$_2$-ascorbic acid (66). For example, peroxynitrite
anion treated *B. subtilis* spores were observed not to release their cortex fragments during germination (61). Those spores initiated germination. However since the cortex was not separated from the core, the size of the core could not increase and the cell formation could not finalize resulting in the loss of viability.

Although these three mechanisms can be listed as possible inactivation pathways, it is highly possible to have more than one mechanism responsible for spore-inactivation by a given disinfectant. In addition, even though the earlier studies showed that the primary lethal effect of sporicidal agents was mainly on the spore protoplast or protoplastic membrane, their activity was largely determined by their ability to produce degradation and/or penetration of both the coat and the cortex permeability barriers (49, 67, 68). Hence, there is still more to know about the inactivation mechanisms of the disinfectant agents.

1.2.4 Reactivity of Ozone and Chlorine Dioxide with Organic Constituents of Spores

The inactivation rates and the shape of inactivation curves are expected to be related to the mass transfer and oxidation rates of the disinfectant gas with the spore structures. Since ozone and chlorine dioxide are strong oxidizers for various organic chemicals, in this section possible organic constituents of spores that can react with ozone and chlorine dioxide are reviewed. The spore structures and major molecules present in these spore structures, which were described in detail in the previous
section can be summarized follows: Spore coat consists mainly of proteins. Cortex contains mainly peptidoglycan, which is a polysaccharide belonging to carbohydrates group. The core membrane consists mainly of proteins and unsaturated fatty acids belonging to lipids group. The spore core contains primarily DNA, proteins and DPA which is a carboxylic acid belonging to lipids group. These main constituents and their reactivity with ozone and chlorine dioxide, which will be discussed in detail in the following sub-sections, are summarized in a tabulated form in Table 1.1.

1.2.4.1 Ozone Reactivity in Water and Gas

Ozone in water reacts with organic substitutes through direct and indirect mechanisms (69). Direct mechanism involves molecular ozone as the major oxidant. Indirect mechanism involves hydroxyl (OH-) radicals that are produced from the ozone interactions with water and substitutes. These radicals are very short-living compounds that have even stronger oxidation mechanisms than that of ozone. In practice, both direct (ozone) and indirect (radicals) oxidation reactions will take place (70). Direct oxidation of organic matter by ozone is a quite selective reaction mechanism during which ozone reacts quickly with organic matter that contains double bonds, unsaturated aromatic and aliphatic compounds (70-72). Ozone oxidizes these compounds through cycle-addition to double bonds. Contrary to those of ozone, hydroxyl radical reactions are largely non-selective. Indirect reactions in an ozone oxidation process can be very complex involving several pathways for formation, reaction and quenching of radicals. The oxidation rates quoted in the literature for
ozonation generally do not separate between these two mechanisms. However in the
gas phase, only direct oxidation by ozone molecule should occur. In addition,
typically ozone in water reacts faster with ionized and dissociated organic compounds
than with the molecular (non-dissociated) type. Since the compounds generally will
be non-dissociated in the gas phase, and the indirect reactions are missing, the
reactivity of ozone in gas phase is expected to be much slower relative to its reactivity
in water phase. In case of high relative humidity presence in the air, ozone may
interact with condensed water and some hydroxyl radical formation may occur.
Therefore, as the relative humidity increases some indirect oxidation reactions might
be expected to occur. This would result in higher reactivity of ozone in the gas form
with increasing relative humidity.

Table 1.1 Main spore constituents and their general reactivity with ozone and
chlorine dioxide

<table>
<thead>
<tr>
<th>Spore Structure</th>
<th>Main Constituents</th>
<th>Significant reaction with O₃</th>
<th>Significant reaction with ClO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore Core</td>
<td>Proteins, enzymes SASP</td>
<td>yes</td>
<td>Only S*</td>
</tr>
<tr>
<td></td>
<td>Lipids, DPA</td>
<td>yes</td>
<td>Only S*</td>
</tr>
<tr>
<td></td>
<td>Ribonucleoids, DNA</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Core Membrane</td>
<td>Proteins</td>
<td>yes</td>
<td>Only S*</td>
</tr>
<tr>
<td></td>
<td>Lipids, unsaturated fatty acids</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Spore Cortex</td>
<td>Carbohydrates, peptidoglycan polymers</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Spore Coat</td>
<td>Proteins</td>
<td>yes</td>
<td>Only S*</td>
</tr>
</tbody>
</table>

S*: Only with proteins that contain sulphur amino acids
1.2.4.2 Ozone Reactivity with Spore Components

Numerous studies have established the ability of ozone to react with amino acids, peptides, and proteins (73-75) both in water and gas phases. Ozone displays a high degree of reactivity toward certain amino acid residues in proteins and unsaturated bonds (76). Ozone attacks the nitrogen atom or the R group or both (75). Rapid oxidation of sulphur-containing groups, which are abundant in microbial enzymes and contain double bonds, may explain rapid inactivation of microorganisms and bacterial spores by ozone (71, 75).

The high reactivity of ozone with proteins and the presence of high protein content in the spore coat suggest that proteins at the spore coat should react with ozone, and thus reduce the amount of ozone that may reach the spore core. The results of recent studies support this idea. The major factor in spore resistance to ozone appears to be the spore coat: \textit{Bacillus} spores with removed coat proteins were rapidly inactivated by ozone in aqueous phase compared to intact spores (75, 77-79). Khadre and Yousef (80) observed that spores of \textit{Bacillus subtilis} treated with aqueous ozone showed heavily disrupted outer spore coats. It should be noted that damages in the spore coat does not affect the spore viability directly. However, it allows the passage of toxic and oxidant molecules to inner structures.

It was reported that ozone reacts with polysaccharides slowly (71, 81) leading to breakage of glycosidic bonds. Perez \textit{et al.} (82) showed that N-acetyl glucosamine, a
compound present in the peptidoglycan spore cortex, was resistant to the action of ozone in aqueous solution at pH 3 to 7.

Ozone reacts slowly with saturated fatty acids. However, unsaturated fatty acids are readily oxidized with ozone and cycle-addition products are formed. The reaction of ozone with lipids, which are part of the core membrane, occurs most exclusively with carbon-carbon double bonds present in unsaturated fatty acid chains (73, 75, 76, 83).

Some recent studies focused on the damage caused by the oxidative agents on the core membrane. Treatment of spores with a variety of oxidizing agents including ozone and chlorine dioxide has been suggested to cause damage to the spore’s inner membrane whose integration is essential for spore viability (78). Oxidative modification of the inner membrane of spores such that this membrane becomes non-functional in the germinated spore, has been pointed out as the leading cause of spore death (65). Most probably, these agents work by causing oxidative damage to key proteins and lipids in the spore’s inner membrane.

This literature review indicates that ozone reaction with peptidoglycan in cortex is slow, yet ozone can damage the protein/lipid structures of the membranes. This review suggests that ozone that can pass the spore coat will diffuse through spore cortex and react with the core membrane. Accordingly, the lethal damage of ozone to spore should occur at this stage.
1.2.4.3 Chlorine Dioxide Reactivity in Water and Gas

It is well known that chlorine dioxide is a very selective oxidant. It has this ability due to a unique one-electron exchange mechanism. Chlorine dioxide reacts only with substances that give off an electron. Chlorine dioxide reacts with sulphuric substances, aromatic amines, nitrogen oxides, cyanides, phenols and aldehydes (86). It does not react with many chemicals including acids, unsaturated fatty acids, carbohydrates, polysaccharides, aliphatic amines, and unsubstituted aromatics, among others. Chlorine dioxide does not dissociate in water and it remains in the molecular form (22). Since chlorine dioxide remains in molecular form in both aqueous and gas phase, it is expected to have the same oxidation mechanism in both phases.

1.2.4.4 Chlorine Dioxide Reactivity with Spore Components

Chlorine dioxide is generally known to react with sulphur-containing amino acids (e.g., cysteine, methionine), and only a few others (e.g., tyrosine, tryptophan) in both aqueous and gas phases while most amino acids do not readily react with chlorine dioxide (86-88).

It appears that chlorine dioxide will not react or react slowly with peptidoglycan or lipids present in cortex and membrane. In addition, it will react with only sulphur containing proteins. The spore coat contains more than 40 types of protein, and many still to be identified. The identified proteins do not contain sulphur.
It is expected to have low percentage of sulphur containing proteins at the remainder of spore coat. Therefore, chlorine dioxide could diffuse through spore coat and cortex without significant reaction, and reach inner parts of the spores easily. Since membrane proteins contain significant amount of sulphur amino acids, it should be expected to have significant reaction at core membrane.

This argument is supported by several studies that reported the damage caused by chlorine dioxide on the cell membranes. In bacterial cell inactivation, it was observed that chlorine dioxide caused inactivation by disruption of outer membrane permeability by altering membrane proteins and lipids (70, 89-91). As it was stated for ozone, treatment of spores with chlorine dioxide was suggested to also cause damage to the spore’s inner membrane (78). For example, chlorine dioxide-treated cells did germinate with the nutrient addition, but they did not metabolize possibly due to damages in their inner membrane (64). Most probably, disinfection by chlorine dioxide occurs by oxidative damage to key proteins in the spore’s core membrane. Therefore, the main oxidation/inactivation by chlorine dioxide should be expected to be at the core membrane of the spores.

Although this research was not designed to study the inactivation mechanisms of the disinfectants, knowing the spore structures and the reactivities of ozone and chlorine dioxide with spore structures will help one to understand and properly interpret the results of the inactivation studies.
1.2.5 Gas Diffusion into the Spores

The transfer of a gaseous disinfectant from bulk form to the spore surface and then through the sub-layers of spores is most likely carried out by molecular diffusion, and hence the transfer process can be presented by the commonly-used Film Theory. A stagnant gas film formation over the spore surface can be visualized where the disinfectant gas diffuses through due to a concentration gradient (Figure 1.2).

Figure 1.2 Gas concentration profile through gas film and spore layers (figure not scaled).
The disinfectant that reaches the spore coat surface will naturally have lower concentration than the bulk gas concentration. The mass flux through the gas film (J, mass/area/time) can be presented by:

\[
J = \frac{D}{\delta_G} \Delta C
\]  

(1.1)

where \( \Delta C \) is the concentration gradient (\( C_{\text{bulk}} - C_{\text{interface}} \)), \( D \) is diffusion coefficient of the gas in air, and \( \delta_G \) is the film thickness. If the gas is reactive with surface molecules (such as coat proteins), a chemical reaction will occur at the surface. Excess disinfectant gas will diffuse through the pores in spore coat, and move inside the spores. Equation 1.1 can also be used to describe diffusion through the spore layers, with the relevant boundary concentrations and the mass transfer coefficient specific to the spore layers. The diffusion through the layers of the spores is expected to be limited by the porosity of the spore components. The studies suggest that the permeability through the spore structures should be similar to a sieving function with decreasing pore dimensions through the layers (50). As the pore dimensions and porosity decrease, total area available for diffusion of the gas will also decrease. In addition, the chemical reaction with reactive sites inside the pores will consume more of the disinfecting gas while it diffuses. Therefore, total amount of gas reaching the spores’ inside components is expected to be less than that diffused into the spores. Furthermore, the higher the reactivity of the gas with the outer spore layers, the less gas to reach the inside core membrane to cause inactivation.
1.2.6 Inactivation Curves

Earlier studies in water showed that the inactivation curves of bacteria, viruses and spores with ozone (18, 20, 92) or chlorine dioxide (19, 93, 94) can generally be characterized by a lag phase or shoulder followed by a phase of pseudo-first-order kinetics. The lag phase was also observed by Ishizaki et al. (5) during inactivation of Bacillus spores with gaseous ozone. The initial lag phase can perhaps be explained by the oxidant demand of the organic constituents of the outer structures of the microorganisms, and the time requirement of disinfectant to diffuse through these outer structures before the inactivation can occur. After the initial lag phase, inactivation evolves exponentially with time until the tailing-off starts to occur. Although some researchers have not reported any tailing-off, most others frequently observed this phenomenon in the survival curves of bacteria, viruses and spores. There are several hypotheses regarding this tailing-off phenomenon (95). Some consider it an experimental error, while some others attribute this to non-homogeneity of bacterial population. However, the majority of the researchers believe that it might be the result of clumping of cells that form a barrier against the disinfectant protecting the insider cells. Still some think that the tailing-off may be caused by an error in counting of the organisms when number of organisms gets too small.

In these previous studies, the initial number of spores was kept the same from experiment to experiment in the same study. The present research was designed to
vary the initial number of organisms during disinfection studies in order to better address the effect of spore population on spore resistance.

Earlier studies in water produced different inactivation rates with ozone for the same species of Bacillus spores or cryptosporidium cysts. Rennecker et al. (96) have observed that different oocyst preparation techniques resulted in different levels of resistance to ozone. Even different stock cultures prepared following the same procedure produced different inactivation curves of B.subtilis spores in ozonated water (92). These differences may be attributed to variations among different strains, purification techniques or combination of these factors. The following factors are identified as possible barriers presented by spores against disinfectants; the thickness, composition, and/or density of the spore coat, the cortex and the inner membrane. A possible explanation for the differences in inactivation kinetics could be the differences in these spore structures. It is known that these factors may change with the changes in the sporulation medium (38, 67, 68, 97, 98). There might also be different factors affecting resistance of spores during growth, sporulation and stock preparation of cultures. These factors can be listed as the type of growth medium, availability of minerals, and the duration of sporulation allowed. This research was designed to include different sporulation conditions to address the effect of these factors on the resistance of spores.
1.2.7 Carrier Material

When properly prepared, anthrax spores can be aerosolized and become available in lethal dosages for inhalation or body contact. The spores may be introduced into the ambient air from opened contaminated packages or envelopes, or through the ventilation systems of buildings. Due to their size, the spores primarily settle onto surfaces after being introduced into the air such as desks, furniture, clothing, walls, rugs, floors, etc. Therefore, it is important to study inactivation of spores on the surfaces of a carrier material rather than in the aerosolized form. In addition, spores placed on the surfaces are much difficult to inactivate than their aerosol form due to mass transfer limitations on the surfaces.

Furthermore, various volatile organic compounds (VOCs) are emitted from different surfaces such as carpeting, furniture, and painted or polished surfaces (99-104). These VOCs can react with the disinfecting agent applied to inactivate the spores. For example, Weschler (99) and Morrison and Nazaroff (101, 102) reported reactions between ozone and various carpet samples at typical indoor ozone concentrations of 30-400 ppb (0.06-0.8 μg/L). The reactions of these very low ozone concentrations have resulted in the production of a variety of VOCs, primarily aldehydes. Molhave et al. (100) showed that several VOCs, mainly terpenes, were emitted from indoor woods. These VOCs were oxidizable by ozone. The type and the concentration of the emissions changed with the raw material, manufacturing process and age of the materials. These reports imply that the type and age of the carrier
material, i.e., the material on which the spores rest, might affect the inactivation of spores by gaseous ozone. If the VOC emission from the materials is considerable, some ozone molecules diffusing the material surface might be consumed by the VOCs before reacting with the spores, thus reducing ozone available for inactivation.

Some carpet types have resulted in emission of formaldehyde when they were exposed to ozone (102). Formaldehyde itself can be used as a gaseous disinfectant for sterilization of hospital rooms and equipment. If formaldehyde is produced by reaction of ozone with certain surfaces on which the spores rest, the inactivation of the spores might be enhanced considerably.

In water, chlorine dioxide is known to be a good disinfectant but a selective oxidant. However, information on capabilities of gaseous chlorine dioxide is very limited. Gaseous chlorine dioxide has been explored for decontamination in the food industry. Inactivation of bacteria and spores on food surfaces and different container materials including epoxy-coated stainless steel (105), green pepper (106), and paper and aluminum foil (21, 107) were reported. Han et al. (108) examined the efficacy of chlorine dioxide for decontamination of \textit{B.thuringiensis} spores on paper, wood, epoxy and plastic surfaces. These researchers used a batch type decontamination chamber where the chlorine dioxide concentration varied with time. The highest inactivation rate of spores was obtained on plastic surfaces followed by epoxy, wood and paper.
These limited data on gaseous ozone and chlorine dioxide indicate that the carrier material may affect the rate of inactivation of spores by either hindering or enhancing it. Hence, the present research addresses this interaction by measuring the inactivation rates of the spores by ozone and chlorine dioxide as they rest on various types of carrier material.

1.2.8 Disinfection Kinetics

The literature on disinfection indicates that the following factors can affect the kinetics and extent of the disinfection processes, in general: (1) the contact time, (2) the concentration and the type of chemical disinfectant, (3) temperature, (4) the number of organisms, (5) the types of organisms, and (6) the nature of the environment. In the case of gaseous disinfection, the following additional factors should be taken into consideration: (7) the condition of the organisms, (8) the hydration level of the organisms, and (9) the layering or clumping of the organisms.

For water applications, the USEPA recommends the use of the first-order Chick-Watson’s law (109, 110) to describe the inactivation kinetics of various disinfection processes (3):

\[
\ln \left( \frac{N}{N_0} \right) = -kCT
\]  

(1.2)
where \( N_0 \) is the initial number or organisms at time \( T=0 \), \( C \) is disinfectant concentration, and \( k \) is a rate constant characteristic of the organism type and water characteristics, e.g., pH and temperature. Despite the fact that both positive and negative deviations from this rule are often reported, the Chick-Watson’s law is the most popular model used for describing disinfection rate in aqueous phase.

Depending on the type of chemical agent, disinfection effectiveness is related to the combined effect of concentration and contact time within limits. This effect has been formulated empirically as follows:

\[
C^nT_p = \text{constant}
\]

where \( n \) is a constant, and \( T_p \) is time required to effect a constant percentage kill. It is common to publish the results as the multiplication of \( C \) and \( T \) (\( \text{CT, mg/L} \times \text{minutes} \)) requirement for a certain level of inactivation in the disinfection studies. The EPA guideline for the required dosage of disinfectant is also listed as the required \( \text{CT} \) (3).

Another form of the expression used in the disinfection studies is the decimal reduction value (D-value). Yokoya & York (97) were first to propose the use of the term “D-value” to express the death rate or the apparent resistance of the organisms. The D-value is defined as the time, in minutes, needed to reduce the population by 90% or 1-log at a constant disinfectant dosage. The D-values are obtained from the slopes of the inactivation curves where \( D=1/\text{slope} \).
The usage of the either concept (CT or D-value) depended on the presentation of the data and the preference of the researchers. Since disinfection studies in air are rare, and mostly reporting the use of only one disinfectant concentration level, the application of the CT concept has been rare in the gaseous studies. Ishizaki et al. (5), who have applied different ozone concentrations in their studies, used the D-value for comparison of the results. However, they stated that the D-values were roughly inversely proportional to the ozone concentration. Therefore, their results indicate that the CT concept might also be applicable in gaseous disinfection.

1.3 RESEARCH OBJECTIVES

The main objective of this research was to investigate the proper operational conditions for the two gaseous disinfectants ozone and chlorine dioxide to inactivate Bacillus subtilis. B.subtilis is a group of spore forming non-pathogenic bacterium that belong to the genus Bacillus, and share the same physiological characteristics as Bacillus anthracis that can cause the infectious anthrax disease. For a better understanding of the factors affecting the inactivation rates of the spores, the effect of several growth and sporulation conditions on the resistance of spores to the disinfectants was also investigated.
Hence, the goals of this research are as follows:

1) Investigation of proper operational conditions for ozone gas inactivation of *Bacillus subtilis* spores.

2) Investigation of proper operational conditions for chlorine dioxide gas inactivation of *Bacillus subtilis* spores.

3) Evaluation of the effects of several growth and sporulation conditions on resistance of spores to the gaseous disinfectant.

4) Comparison of ozone and chlorine dioxide in terms of inactivation efficiency, and ease and effectiveness of applicability for gaseous remediation of contaminated spaces.

5) Investigation of ozone and chlorine dioxide activities in water on *Bacillus subtilis* spores in an attempt to evaluate the disinfection mechanisms of ozone and chlorine dioxide.

The specific objectives to meet the goals are as follows:

For goal 1 and 2:

1. Determination of the proper ozone (goal 1) and chlorine dioxide (goal 2) concentrations, air humidity level, and the degree of pre-hydration of spores for effective inactivation of spores.

2. Evaluation of the effect of different surface materials on inactivation of spores.
3. Evaluation of possible collateral damage and its extent, if any, on sensitive materials resulting from corrosive effects of ozone or chlorine dioxide.

For goal 3:

Evaluation of the effect of the following on the resistance of spores and shape of the inactivation curves:

1. Two different organic carbon sources for growth medium.

2. The concentration of Fe$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$, the main cations required in the growth medium.

3. The difference between growing in liquid broth medium and on solid agar medium.

4. The desiccation time.

5. The initial number of spores.

For goal 4:

1. Comparison of the inactivation rates obtained by ozone and chlorine dioxide as disinfectant under similar conditions

2. Assessment of overall performances of both disinfectants in terms of inactivation efficiency, ease of application, gas production and clean-up after
remediation, possibility of collateral damage on the remediation site, and collection of required information

For goal 5:

1. Determination of the inactivation efficiency of the ozone and chlorine dioxide in aqueous phase.

2. Comparison of the inactivation rates and curves obtained by ozone and chlorine dioxide in aqueous and gaseous phases and assessment of the inactivation mechanisms of both disinfectants.

1.4 OUTLINE OF RESEARCH

In order to achieve the objectives of this research, a series of experiments were planned, designed and conducted. The experimental set-up, procedure and analytical methods used were described in Chapter 2. In Chapter 3, all the results obtained from these experiments were presented. The discussion of the data and results were provided in Chapter 4. Finally, major findings of this research are summarized in Chapter 5.
CHAPTER 2

EXPERIMENTAL SET-UP AND METHODS

2.1 EXPERIMENTAL SET-UP

Two different test chambers were designed and constructed for ozonation and chlorine dioxide studies.

The ozonation test chamber was made of plexiglas to allow transparency for observation. Figures 2.1 and 2.3(a) show the schematic and the photograph of the ozonation test system, respectively. The chamber was 76 cm long, 56 cm deep and 30 cm wide (volume 128 L), sealed perfectly. The system was constructed and parts were assembled specifically for this study by Pure-O-Tech Inc. of Escondido, CA. The system had its own air compressor, Sequlal oxygen concentrator and Pure-O-Tech ozone generator. The generator was capable of producing 4% ozone in gas. Three gas streams were directed to the chamber to create the desired conditions in the chamber in terms of ozone concentration, humidity level, and the total gas flow through the chamber. These gas streams were (1) the ozone gas stream from the ozone generator, (2) the air stream from the air compressor, and (3) the humidified air stream which is produced by bubbling air into sterile water. The flow of each line was controlled with
in-line flow-meters. The influent gas stream was distributed evenly in the chamber through the diffuser placed on the entrance side of the chamber. The influent and effluent ozone gas concentrations were monitored by a UV spectrophotometer. The relative humidity (RH) and temperature were measured by the humidity/temperature sensor, which was placed inside the chamber. Two sample entry windows at the top of the chamber were designed to allow withdrawal of the test samples periodically during the experiments without affecting the exposure of other samples. The effluent from the chamber was passed through an ozone destruction system before being released to the atmosphere.

Figure 2.1 Schematic diagram of ozonation test chamber.
The chlorine dioxide gas test chamber was similar to the ozone test chamber (Figure 2.2 and 2.3(b)). It was also made of plexiglas to allow transparency for observation, but it was covered with a special filter to minimize the penetration of light and consequently minimize the destruction of chlorine dioxide. This chamber was 76 cm long, 45 cm deep and 30 cm wide (volume 103 L), and was also sealed perfectly. This chlorine dioxide system was also constructed and the parts were assembled specifically for this study by Pure-O-Tech Inc. of Escondido, CA. The system contained a CDG bench-scale chlorine dioxide generator that used chlorine gas and sodium chlorite for production of chlorine dioxide gas as shown by the following reaction:

\[
\text{Cl}_2 + 2 \text{NaClO}_2 \rightarrow 2 \text{ClO}_2 + 2 \text{NaCl}
\]  

The system had its own air compressor, CDG chlorine gas tank, and CDG sodium chlorite cartridge (CDG Technologies, Bethlehem, PA). The other auxiliary parts were same as the ozone test chamber. Both chambers were operated by following the same procedures.

### 2.2 EXPERIMENTAL PROCEDURE

In this section the experimental procedure followed to conduct inactivation experiments with ozone and chlorine dioxide is presented.
Figure 2.2 Schematic diagram of chlorine dioxide test chamber

Figure 2.3 Pictures of (a) ozone and (b) chlorine dioxide test chambers
2.2.1 Ozone Inactivation Experiments

The ozone inactivation experiments were conducted in the ozonation test chamber at continuous flow, steady-state conditions at the room temperature of 21-22°C. The RH was controlled by changing the proportion of the two inlet air streams. The influent and effluent ozone gas concentrations were monitored by a UV spectrophotometer by measuring the light absorbance at 258 nm. Ozone gas concentration was controlled by changing the proportion of the inlet ozone and air gas streams. When the desired experimental conditions were reached, and the steady-state conditions were achieved, the test samples were placed into the chamber through two sample entry windows at the top of the chamber, and the chamber was completely sealed. The samples were withdrawn periodically during the test out of the chamber in duplicates, and were analyzed for spore viability.

2.2.2 Chlorine Dioxide Inactivation Experiments

The chlorine dioxide inactivation experiments were conducted in the chlorine dioxide test chamber at continuous flow, steady-state conditions at the room temperature. The experimental procedure was identical to the ozonation test procedure except that ozone was replaced by chlorine dioxide. The influent and effluent chlorine dioxide gas concentrations were monitored by a UV spectrophotometer by measuring the light absorbance at 360 nm.
2.3 SAMPLE PREPARATION

In this section, the procedure followed to prepare the experimental samples for the inactivation experiments and for data gathering is presented.

2.3.1 Preparation of Spore Cultures

*Bacillus subtilis* sp. ATCC 6633 was obtained from the Microbiology Laboratories of the Biology Department of SDSU. The strain was grown in Tryptic Soy Broth (TSB) with Schaeffer sporulation media (111) (K$_2$HPO$_4$, 10.5 g/L; KH$_2$PO$_4$, 3.5 g/L; MgSO$_4$.7H$_2$O, 0.05 g/L; CaCl$_2$, 0.05 g/L; MnCl$_2$.4H$_2$O, 0.005 g/L; Fe$_2$(SO$_4$)$_3$.7H$_2$O, 0.005 g/L). One week was allowed for growth and sporulation with moderate aeration at 30°C. The culture, in the form of vegetative cells and spores, was harvested and heat shocked at 65°C for 15 minutes to kill the vegetative cells. Afterwards, the suspension was centrifuged, washed and resuspended in distilled water. The suspension was filtered through a 2 μm glass fiber filter paper to remove the debris. The remaining cells (spores) were centrifuged, washed and resuspended in distilled water for five times. The final stock spore solution was stored in a refrigerator at 3°C. Necessary dilutions were applied to bring stock spore solution population to the desired initial level.
2.3.2 Preparation of Test Samples

Spore solutions were placed on test strips of various materials of common use in offices and residences, e.g., glass, carpet, paper, vinyl floor material, and hardwood. Microscope slides served as glass carrier. Two different carpet samples were tested; the first one was a new loop pile carpet, and the second one was part of an old used cut pile carpet. Parts from an old office desk were used as finished hardwood samples. Two different types of new vinyl floor tiles were obtained from a hardware store. One of the vinyl sample had printer top layer and vinyl mid and back layer (brown vinyl). The second vinyl sample was solid excelon vinyl tile (black vinyl). Regular office print paper was used as the paper sample.

Glass strips were used as the base test material since the demand of glass for ozone/chlorine dioxide is expected to be minimal compared to other materials. In addition, microorganisms on impervious surfaces may be harder to inactivate than pervious ones (5, 112). After placing and distributing 50 μL of spore solution on each strip surface, the strips were dried at room temperature in a safety cabinet for three hours, and conditioned in desiccators for at least three days. The relative humidity in the desiccators was maintained at less than 1% by using silica gel. When pre-conditioning of the samples was required, a constant 90% humidity level was maintained by using a saturated salt solution of BaCl₂.
2.3.3 Recovery of Spores

In order to quantify the number of viable spores on the test strips, the strips were removed from the chambers, and were transferred into sterile tubes that contained 50 mL of physiological phosphate buffer solution. Ultrasonication was applied to transfer the spores to the buffer solution. The physiological phosphate buffer solution contained 10 mM $\text{K}_2\text{HPO}_4$, 1.0 g/L $\text{MgSO}_4$, 0.05% Tween-20, and 1 mM sodium thiosulfate to quench ozone or chlorine dioxide. Various control experiments were performed to determine, although highly unlikely, if there would be any inactivation due to sonication with a Branson B-220 sonication bath. These experiments were also used to determine the sonication time required to recover a minimum of 90% of spores from the test strips.

2.3.4 Determination of Number of Spores

The Spread Plate Viable Counting Method (113) was used to determine the number of spores transferred by sonication into the buffer solution. Required serial dilutions were applied to the sample solutions by using the sterile phosphate buffer solution as the diluent. Inoculum samples of 0.1 mL from each dilution tube were spread on Tryptic Soy Agar (TSA) plates. The agar plates were incubated at 30°C for 16-24 hours. The colonies formed on agar surface were counted. When the number of spores in the sample was very low (less than 100 c.f.u./mL), the Pour Plate Viable Counting Method (113) was used instead. Twenty five mL of the liquid TSA solution,
which was kept at 45°C, was put in a test tube; 1mL or 10 mL of samples containing the spores was added to the TSA solution. After mixing the contents of the test tube, the solution was poured into a petri dish. Solidified agar plates were incubated at 30°C for 24-48 hours after which the colonies formed inside the agar medium were counted.

2.4 AQUEOUS PHASE INACTIVATION STUDIES

Inactivation of spores by ozone and chlorine dioxide in aqueous phase was investigated in phosphate-buffered distilled water in batch mode. These studies were conducted in 500 mL Erlenmeyer flasks using 400 mL of water. The flasks were washed with concentrated ozone or chlorine dioxide stock solution to minimize oxidant demand of the container.

First, a saturated stock ozone solution was obtained by bubbling ozone gas into distilled water in a container. In an Erlenmeyer flask, a buffer solution was prepared by addition of 4 mL of 1 M phosphate solution into 250 mL distilled water. The contents of the flask were mixed continuously with a magnetic stirrer. A required amount of stock ozone solution was added into the flask, and the total volume was brought to 400 mL by additional distilled water. The pH of the final solution was 7.0. The experiments were conducted at room temperature of 21-22°C temperature. After initial ozone concentration in the flask was adjusted to the desired level, a known
volume of spore solution was added to the flask to have an initial spore concentration of about $10^6$ spores per mL of water. The addition of the spores was marked as time zero. Samples were then taken periodically for determination of the number of viable spores and the residual ozone concentration in water. The samples collected for spore determination were taken into sterile test tubes. The test tubes contained 0.1 mL of 1 M sodium thiosulfate solution to immediately quench ozone or chlorine dioxide present in water to stop further inactivation of spores.

The saturated chlorine dioxide solution was obtained in distilled water in accordance with the Standard Methods (113) using a series of solutions in gas washing bottles. Gaseous chlorine dioxide was prepared by slowly adding dilute sulfuric acid to a sodium chlorite solution. Contaminants such as chlorine are removed from the gas stream by a sodium chlorite scrubber. The gas is passed into distilled water to obtain a pure aqueous solution of chlorine dioxide. The chlorine dioxide tests were conducted following a procedure similar to that of ozone tests.

2.5 ANALYTICAL METHODS

Ozone concentration in gas streams was measured by a spectrophotometer (Shimadzu UV-1601) at the wavelength of 258nm. Chlorine dioxide concentration in gas streams was measured at 360nm with the same spectrophotometer. The spectrophotometer was calibrated by the Potassium Iodide (KI) method. This method
involves dissolution of ozone or chlorine dioxide gas in a 2% potassium iodide solution, and the titration of the iodine formed with standard sodium thiosulphate solution under acidic conditions (113). The calibrations obtained were in accordance with the published molar extinction coefficients \((a)\) of both gases: \(a\) (ozone): 3024 \((\text{M} \cdot \text{cm})^{-1}\) at 258nm (115), and \(a\) (chlorine dioxide): 1150 \((\text{M} \cdot \text{cm})^{-1}\) at 360nm (114).

Ozone concentration in the liquid phase was determined by the Indigo Method (113). This method uses the decolorization of indigo trisulfonate upon its reaction with ozone. Indigo trisulfonate is a blue dye that absorbs light at 600nm. Chlorine dioxide concentration in the liquid phase was determined by measuring the light absorbance at 360nm by a spectrophotometer. The spectrophotometer was calibrated by the Potassium Iodide method for both reactants in the liquid phase.
CHAPTER 3

RESULTS

3.1 INTRODUCTION

In this chapter, the results of the experiments are presented. The results include the control experiments that were conducted to determine the hydrodynamic characteristics of the test chambers as well as the effect of sonication on B. subtilis sp. spore viability and recovery before starting the inactivation tests. The ozone and chlorine dioxide inactivation tests were performed to determine the effect of ozone and chlorine dioxide gas concentration, relative humidity of the air, pre-hydration of the dry spores, and carrier materials on the rate of inactivation of the spores. The results of the study on the extent of the possible collateral damage induced by ozone and chlorine dioxide on different materials are also presented. In addition to the gas phase studies, the results of the inactivation of spores in aqueous phase with both disinfectants are presented. Finally, this chapter is concluded with the results of the effect of growth and sporulation conditions tests on the inactivation rate of the spores.
3.2 CONTROL EXPERIMENTS

In this section, the results of the control experiments performed by using the test chambers and the spore solutions are presented.

3.2.1 Hydrodynamic Characterization of the Ozonation Test Chamber

Flow distribution inside the ozonation chamber was analyzed by both visual and tracer tests. A constant flow of smoke was introduced to the chamber in order to observe the pattern of smoke distribution inside the chamber. Visually, the smoke was distributed uniformly throughout the entire chamber in a period equivalent to 3-4 residence times (RT). RT is defined as the volume of the chamber divided by the flow rate of the gas mixture through the chamber. The flow pattern was also tested by using ozone gas itself as the tracer. Ozone gas was introduced into the chamber at a constant flow rate and concentration. The inflow and effluent ozone concentrations were monitored by a UV spectrometer as a function of time. Ozone breakthrough curves were obtained at various RT values. Numerical data analysis has shown that the flow regime was very close to complete mix conditions (CSTR) as normalized effluent concentration curves overlapped with ideal CSTR curves (Figure 3.1). The effluent gas concentration became equal to the influent gas concentration after a period equivalent to 4 RT. These tests provided enough evidence that complete mixing conditions were maintained in the chamber, so that all test strips placed in the
Figure 3.1 Ozone test chamber break through curves.

chamber were exposed to the same levels of ozone. Furthermore, the same ozone levels were reached in the chamber and the effluent as in the influent gas stream after a period of 4 RT. This information was important in reaching the steady-state conditions before exposing the spores to ozone in the chamber.

3.2.2 Hydrodynamic Characterization of the Chlorine Dioxide Test Chamber

Flow distribution inside the chlorine dioxide test chamber was analyzed by using chlorine dioxide gas itself as the tracer. Chlorine dioxide gas was introduced into the chamber at constant flow rate and concentration. The inflow and effluent chlorine dioxide gas concentrations were monitored by a UV spectrometer. Chlorine
dioxide breakthrough curves were obtained at various RT values. Numerical data analysis has shown that the flow regime of this chamber was also very close to a CSTR as normalized effluent concentration curves overlapped with ideal CSTR curves (Figure 3.2). The effluent from the chamber stabilized after a period equivalent to 4 RT. This information was important in reaching the steady-state conditions before exposing the spores to chlorine dioxide in the chamber.

Figure 3.2 Chlorine dioxide test chamber breakthrough curves.
3.2.3 Effect of Ultrasonication on Inactivation of Spores

Although sonication was observed to induce no inactivation on spores according to an earlier study (6), the following control experiment was carried out to determine whether the conditions applied in this study would induce any inactivation. A 50μL volume of stock spore solution was diluted into 50 mL physiological buffer solution to which ultrasonication was applied. At ½-hour time intervals, samples were taken from the solution and subjected to the procedure designed for counting the number of spores. The results are shown on Figure 3.3. The data points on the graph are the average of duplicate runs, and the error bars denote one standard deviation from the mean value. No significant differences were observed among the measurements indicating that ultrasonication has not affected the activity of spores during 2 hours of sonication.

![Figure 3.3 Number of spores versus ultrasonication time.](image)
3.2.4 Ultrasonication Time Required for Recovery of Spores

The efficacy of sonication for dislodging spores from the strips was determined by carrying out the following control experiments. Strips of different material that were conditioned in desiccators were placed in 50mL tubes containing physiological buffer solution. Ultrasonication was applied to the tubes. At ½-hour time intervals, samples were taken from the solution and the number of spores was counted. The results obtained with the glass strips are shown on Figure 3.4. As seen from the figure, the percent recovery improved with time to reach about 90%. No significant increase in recovery was observed beyond 90 minutes. Therefore, 90 minutes of sonication was applied for recovery of the spores from the glass, vinyl and hardwood strips, within which about 90% of the spores were recovered. The recovery from the carpets and paper strips did not exceed 50% but the recovery ratio was consistent throughout the experiments.

![Figure 3.4 Percentage of spores recovered from glass strips versus ultrasonication time.](image-url)
3.3 OZONE INACTIVATION EXPERIMENTS

In this section, the results of the ozonation experiments with *B. subtilis* sp. spores are presented.

3.3.1 Effect of Ozone Concentration on Inactivation

Spores of *B. subtilis* sp. (stock spore solution, S1) dried on glass strips and preconditioned at less than 1% RH were exposed to different ozone concentrations at 90% RH and ambient temperature of 22°C. The experimental results presented in Figure 3.5 depict the ratio of number of live spores at the time of sampling to the initial number of live spores in log units over the exposure time. Duplicate samples were drawn each time, and each experiment was conducted in duplicates. In figures, each data point represents the average of four readings, and the error bars correspond to one standard deviation in measurements.

The results in Figure 3.5 show a short lag phase followed by an exponential decrease in the number of surviving spores. The lag phase shortens considerably with increasing ozone concentration. The exponential decrease seems to be composed of two different rates; a steep decrease followed by tailing off. A similar behavior in the inactivation curves of bacteria, viruses and spores in water are frequently reported in the literature (95). The results presented in Figure 3.5 also show that increasing the
ozone concentration from 1 to 3 mg/L increased the rate of inactivation of spores. Yet, beyond 3 mg/L, no additional rate increase was obvious.

The increase of inactivation rate with concentration can be seen more clearly by using a CT (concentration × time) graph. The ozone concentrations were multiplied with time values to obtain the inactivation curve shown in Figure 3.6. Data obtained with 1, 2 and 3 mg/L ozone all follow a single inactivation curve, while the data of 10 mg/L ozone fall on a separate curve with higher CT values. Under the experimental conditions presented in Figure 3.6, the CT concept was found applicable for ozone concentrations less than 3 mg/L.

Yokoya & York (97) proposed to use a term referred to as “D-value” to express the apparent resistance of the organisms. It was defined as the time needed to reduce the population by 90% or 1-log at a constant disinfectant dosage. The D-values calculated for both stages of inactivation curves are listed in Table 3.1. In order to determine whether significant differences in inactivation rates existed between the two stages of the same and different treatments, analysis of variance (ANOVA) was performed using t-test with 95% confidence interval (p=0.05). The ANOVA results showed that there was significant difference between first and second stages of the each treatment. As ozone concentration was increased from 1 mg/L to 3 mg/L, the D-values for both stages decreased significantly, while there was no significant difference between the corresponding D-values for 3 mg/L and 10 mg/L.
Figure 3.5 Effect of ozone concentration on inactivation of *B. subtilis* sp. spores at 90% RH and 22°C.

Figure 3.6 The CT curve for ozone at 90% RH and 22°C.
Table 3.1 D-values obtained with different ozone concentrations at 90% RH for inactivation of *B. subtilis* sp. spores on glass strips at 22°C.

<table>
<thead>
<tr>
<th>Ozone concentration (mg/L)</th>
<th>First stage D-value (min)</th>
<th>Second stage D-value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>79</td>
<td>360</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>226</td>
</tr>
<tr>
<td>3</td>
<td>43</td>
<td>136</td>
</tr>
<tr>
<td>10</td>
<td>47</td>
<td>98</td>
</tr>
</tbody>
</table>

3.3.2 Effect of Relative Humidity

*B. subtilis* sp. spores conditioned at less than 1% RH were exposed to 10 mg/L ozone concentration at different RH values. The results are presented in Figure 3.7. All inactivation curves showed two-stage behavior with the D-values increasing with decreasing RH (Table 3.2). The duration of the initial lag phase also decreased with increasing humidity. The positive effect of RH on inactivation efficiency was evident in our results. The D-values obtained at 95% RH were almost four times smaller than the values obtained at 70% RH.

Table 3.2 D-values versus RH for inactivation of *B. subtilis* sp. spores on glass strips at 10 mg/L ozone and 22°C.

<table>
<thead>
<tr>
<th>Relative humidity (%)</th>
<th>First stage D-value (min)</th>
<th>Second stage D-value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>107</td>
<td>274</td>
</tr>
<tr>
<td>80</td>
<td>56</td>
<td>149</td>
</tr>
<tr>
<td>90</td>
<td>47</td>
<td>98</td>
</tr>
<tr>
<td>95</td>
<td>26</td>
<td>77</td>
</tr>
</tbody>
</table>
3.3.3 Effect of Pre-hydration

In order to investigate the effect of pre-hydration of spores on their inactivation rate by ozone, spores of *B. subtilis* sp. were first dried on glass strips. Then, these strips were exposed to pre-hydration at 90% RH for periods of 3-hour and 24-hour. They were subjected to inactivation in the chamber at 10 mg/L ozone and 90% RH following the pre-hydration. The results were compared in Figure 3.8 to the results obtained without pre-hydration. The D-values are presented in Table 3.3. Pre-hydration eliminated the initial lag phase and resulted in higher initial inactivation rate.
(first stage) only. Apparently, 3-hour pre-hydration was sufficient. No further advantage was observed with longer pre-hydration.

Figure 3.8 Effect of pre-hydration at 90% RH on inactivation of \textit{B.subtilis sp.} spores at 10 mg/L ozone concentration, 90% RH and 22°C.

Table 3.3 Effect of pre-hydration on the D-values at 10 mg/L of ozone concentration, 90% RH and 22°C.

<table>
<thead>
<tr>
<th>Pre-hydration</th>
<th>First stage D-value (min)</th>
<th>Second stage D-value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>47</td>
<td>98</td>
</tr>
<tr>
<td>3 hours at 90% RH</td>
<td>31</td>
<td>83</td>
</tr>
<tr>
<td>24 hours at 90% RH</td>
<td>27</td>
<td>87</td>
</tr>
</tbody>
</table>
3.3.4 Effect of Carrier Materials Surface

Spores placed on the surface of different test materials were exposed to 10 mg/L ozone at 90% RH. The inactivation curves and the D-values obtained for these experiments are presented in Figure 3.9 and Table 3.4, respectively. The inactivation rates of spores on glass, vinyl floor tiles and paper were not significantly different from each other. The results on the two vinyl types were similar, thus they are shown as one sample.

Two different carpet types tested in this study produced completely different results from each other. The new loop pile carpet sample has resulted in much faster spore inactivation than the glass surface. The used cut pile carpet sample, on the other hand, resulted in slower inactivation than glass. Compared to the glass surface, slower inactivation was observed on the finished hardwood sample as well. Over the duration of the experiment, the inactivation curves obtained on these two surfaces - the cut pile carpet and the hardwood - did not exhibit the two stages observed for other surfaces.

Table 3.4 D-values obtained with different carrier materials for inactivation of *B. subtilis* sp. spores with 10 mg/L ozone concentration at 90% RH and 22°C.

<table>
<thead>
<tr>
<th>Carrier material</th>
<th>First stage D-value (min)</th>
<th>Second stage D-value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>47</td>
<td>98</td>
</tr>
<tr>
<td>Vinyl floor material</td>
<td>65</td>
<td>103</td>
</tr>
<tr>
<td>Office paper</td>
<td>64</td>
<td>98</td>
</tr>
<tr>
<td>Carpet-loop pile</td>
<td>24</td>
<td>89</td>
</tr>
<tr>
<td>Carpet-cut pile</td>
<td>109</td>
<td>109</td>
</tr>
<tr>
<td>Hardwood</td>
<td>174</td>
<td>174</td>
</tr>
</tbody>
</table>
Figure 3.9 Effect of carrier material on inactivation of *B. subtilis* sp. spores at 10 mg/L ozone concentration, 90% RH and 22°C.
For the loop pile carpet, the enhancement in the inactivation rate compared to the glass surface might be the result of fumigative effect of chemicals produced from the reaction of ozone with the carpet surface. We performed additional experiments in order to test the hypothesis that reaction by-products of ozone with the loop pile carpet can, in fact, function as a disinfectant and inactivate the spores. Samples of loop pile carpet strips were ozonated for 4 hours and kept in desiccators for 24 hours. Then spores were placed on both ozonated and non-ozonated carpet strips and dried in desiccators for 3 days. The recovery of spores from these carpet strips as a function of sonication time is presented in Figure 3.10. More than 90% less live spores were recovered from the pre-ozonated carpet strips compared to the non-ozonated ones at the end of 2-hours of sonication. This is equivalent to inactivation of spores by more than 1-log on pre-ozonated carpet strips.

![Figure 3.10 Recovery of spores from pre-ozonated and non-ozonated loop-pile carpet strips.](image-url)
3.3.5 Physical Effect of Ozone Exposure on Materials

There was no apparent damage or discoloration observed on the surface of the carrier materials tested after 4 hours of ozonation. There was no difference between the materials before and after the ozonation that can be determined visually or though touch.

In order to assess the possible damage induced by ozone, several different types of materials were exposed to 10 mg/L ozone at 90% RH for longer periods of time. These materials included a calculator, floppy discs, compact discs, a zip disc, and several types of tygon and teflon tubings. In case of a bioterrorism attack that may target high-value facilities, it may not be so much the hardware that is of concern but rather the stored data which may be irreplaceable (6). Following a decontamination application, one would like to have electronic devices and storage media still operate so that essential data can at least be recovered. Hence in this study, these simple devices were used as substitutes for more complex electronic equipment.

Floppy discs were first to fail during long term exposure to high concentration of ozone. After 9 hours of exposure, bad sectors started to form on disc surfaces. They completely failed after 11 hours. The zip disc started to fail after 20-23 hours of exposure. The calculator was not affected during the first 30 hours. Some key errors were observed in 34 hours, and none of the keys worked after 40 hours. The compact disc was not affected even after 40 hours exposure.
There was no apparent change in teflon tubings after 40 hours of ozone exposure. But tygon tubings started to show color change after 20 hours, and their elasticity increased due to reactions with ozone. However, they were far from complete failure even after 40 hours of exposure.

3.3.6 Effect of Stock Spore Solution on Inactivation Rate

During the course of inactivation studies with ozone, only one spore stock solution, S1 with about $8 \times 10^7$ c.f.u./mL, was used for all experiments. However, to see the effect of different spore stock solutions, another stock solution with higher spore population was prepared. This new solution, S2, contained about $2 \times 10^9$ c.f.u./mL. The higher population was obtained by having larger volumes of growth solutions and by increasing the density by centrifugation. The inactivation curves of these stock solutions were obtained by exposing the test strips to 10 mg/L ozone at 90% RH and 22°C. In addition, two dilutions of S2, 1/10 dilution (S2-Dil 1) and 1/100 dilution (S2-Dil 2), were tested as different stock solutions (Figure 3.11).

The slowest inactivation was observed with S2 stock which had the highest microbial density. The shape of the inactivation curve obtained with S2 stock was different than others. It seems like some degree of inactivation mixed with a very long lag phase. The lag phase with S2 seems to last about 3 hours, but since it lasted very long, inactivation of surface spores might occur during this time. Therefore, the first part of the inactivation curve has a slower rate which was followed by an exponential
phase with higher inactivation rate. S2-Dil 1 and S1 had spore densities close to each other, and they produced similar first phase inactivation rates. However, slower second phase inactivation was observed with S1. Highest inactivation rate was observed with S2-Dil 2 as it had the lowest spore density.

3.4 CHLORINE DIOXIDE INACTIVATION EXPERIMENTS

In this section, the results of the chlorine dioxide inactivation experiments with *B.subtilis* sp. spores are presented.
3.4.1 The Need for a New Spore Stock Solution

When the stock spore solution S1 (about $8 \times 10^7$ c.f.u./mL) was used for the first chlorine dioxide experiment, complete inactivation was obtained in less than 10 minutes with 6 mg/L (2200 ppm) chlorine dioxide at 90% RH (Figure 3.12). It was obviously not practical to continue experiments with this stock solution. A new stock solution with higher number of spores was prepared (about $2 \times 10^9$ c.f.u./mL), and this spore solution (S2) was used for all chlorine dioxide experiments. Due to higher density of S2, the rate of inactivation with this solution was observed to be slower than that of S1.

![Figure 3.12](image-url)  
*Figure 3.12 The inactivation curves of two stock spore solutions with 6 mg/L chlorine dioxide at 90% RH and 22°C.*
3.4.2 Effect of Chlorine Dioxide Concentration on Inactivation

Spores of B.subtilis sp. (stock spore solution S2) dried on glass strips and preconditioned at less than 1% RH were exposed to different chlorine dioxide concentrations at 90% RH and 22°C. The experimental results are presented in Figure 3.13.

The inactivation curves obtained did not show any lag phase during the initial sampling interval. A steep exponential decay in the number of spores observed with the start of the experiment followed by a tailing-off with reduced rate. Complete inactivation was observed for each experiment as no growth was observed in subsequent sample after the last data shown.

The D-values calculated for both stages of inactivation curves are listed in Table 3.5. The ANOVA results showed significant differences between first and second stages of each treatment. Furthermore, as chlorine dioxide concentration was increased from 1.5 to 6 mg/L, the D-values of both stages decreased significantly.

<table>
<thead>
<tr>
<th>Chlorine dioxide concentration (mg/L)</th>
<th>Relative humidity (%)</th>
<th>First stage D-value (min)</th>
<th>Second stage D-value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>90</td>
<td>7.9</td>
<td>40.4</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>5.1</td>
<td>37.0</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>3.5</td>
<td>16.5</td>
</tr>
<tr>
<td>1.5</td>
<td>75</td>
<td>56.9</td>
<td>91.0</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>18.5</td>
<td>54.4</td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td>13.1</td>
<td>34.9</td>
</tr>
</tbody>
</table>
Figure 3.13 Effect of chlorine dioxide concentration on inactivation of *B.subtilis* sp. spores at 90% RH and 22°C.

Figure 3.14 Effect of chlorine dioxide CT on inactivation of *B.subtilis* sp. spores at 90% RH and 22°C.
Data were analyzed to determine if the CT concept was applicable for chlorine dioxide in gas phase. The measured concentrations were multiplied with corresponding time values to obtain the inactivation curves shown on Figure 3.14. As seen from the figure, the data could not be presented by a single curve. As concentration was increased, higher CT value was required for the same level of inactivation. This result showed that under the current experimental conditions, CT concept could not apply.

In order to compare our results with the remediation studies applied for the clean-up of the facilities after 2001 anthrax attacks (22), additional experiments were carried out at 75% RH. The inactivation curves obtained were similar in shape to those obtained at 90% RH but the rates were slower. The concentration effect on the inactivation rate was clearer as the inactivation curves were more distant from each other (Figure 3.15). The inactivation versus CT graph shown on Figure 3.16 indicates that the data fit a single curve. The data sets were not significantly different from each other statistically. Hence, the results showed that within the same concentration range the inactivation of spores followed the CT concept at 75% RH, but not at 90% RH.

3.4.3 Effect of Relative Humidity

*B. subtilis* sp. spores conditioned at less than 1% RH were exposed to 6 mg/L chlorine dioxide concentration at different RH values. The results are presented in Figure 3.17. The positive effect of RH on inactivation efficiency was also observed
Figure 3.15 Effect of chlorine dioxide concentration on inactivation of *B. subtilis* sp. spores at 75% RH and 22°C.

Figure 3.16 Effect of chlorine dioxide CT on inactivation of *B. subtilis sp.* spores at 75% RH and 22°C.
with chlorine dioxide. The inactivation rate increased as relative humidity in the air was increased. All inactivation curves showed two-stage behavior. D-values decreased with increasing RH (Table 3.6).

### Table 3.6 D-values obtained with 6 mg/L of chlorine dioxide concentration and different RH for inactivation of *B. subtilis* sp. spores on glass strips at 22°C.

<table>
<thead>
<tr>
<th>Relative humidity (%)</th>
<th>First stage D-value (min)</th>
<th>Second stage D-value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>32.2</td>
<td>237.4</td>
</tr>
<tr>
<td>75</td>
<td>13.1</td>
<td>34.9</td>
</tr>
<tr>
<td>90</td>
<td>3.5</td>
<td>16.5</td>
</tr>
</tbody>
</table>

![Figure 3.17 Effect of relative humidity on inactivation of *B. subtilis* sp. spores at 6 mg/L chlorine dioxide concentration and 22°C.](image-url)
The change in the D-values with change in humidity was substantial. For example, the D-values obtained at 90% RH were less than one-tenth of the values obtained at 65% RH. Complete inactivation was observed at both 75% and 90% RH, but at 65% RH only 2-log inactivation was observed within 5 hours. This observation indicated the need for a certain level of humidity below which inactivation is not feasible.

### 3.4.4 Effect of Pre-hydration

In order to investigate the effect of pre-hydration of spores on the inactivation rate by chlorine dioxide, spores of *B. subtilis* sp. that were dried on glass strips were subjected to pre-hydration at 90% RH for periods of 3-hour and 24-hour. These strips were then subjected to inactivation in the chamber at 6 mg/L chlorine dioxide and 90% RH. The inactivation curves obtained from pre-hydrated and non-hydrated spores did not differ from each other significantly (data not shown).

### 3.4.5 Effect of Carrier Materials Surface

Spores placed on the surface of different test materials were exposed to 6 mg/L chlorine dioxide at 90% RH. The inactivation curves and the D-values obtained for these experiments are presented in Figure 3.18 and Table 3.7, respectively.
Figure 3.18 Effect of carrier material on inactivation of *B. subtilis* sp. spores at 6 mg/L chlorine dioxide, 90% RH and 22°C.
Table 3.7 D-values obtained with different materials for inactivation of *B. subtilis* sp. spores with 6 mg/L chlorine dioxide concentration at 90% RH and 22°C.

<table>
<thead>
<tr>
<th>Carrier material</th>
<th>First stage D-value (min)</th>
<th>Second stage D-value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>3.5</td>
<td>16.5</td>
</tr>
<tr>
<td>Carpet-loop pile</td>
<td>10</td>
<td>31</td>
</tr>
<tr>
<td>Office paper</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Brown vinyl floor tile</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Carpet-cut pile</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Black vinyl floor tile</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Hardwood</td>
<td>209</td>
<td>209</td>
</tr>
</tbody>
</table>

The inactivation rates of spores on glass surface were significantly higher than all other tested materials. The use of other materials resulted in significant decrease and delay in inactivation in comparison to glass. Different rates of inactivation were observed with two types of vinyl floor materials, which had resulted in same inactivation rates with ozone gas. The brown vinyl tile had printer top layer while the black vinyl tile had no top layer. The spores on brown vinyl and paper strips showed similar inactivation levels, as it was the case with ozone.

The shape of the inactivation curves was different for different materials. The inactivation curves with office paper and cut pile carpet showed about 10 minutes of lag period during which the inactivation was minimal. Following the lag period, one-phase exponential decay was observed without the tailing-off. Furthermore, complete inactivation was obtained with the sample taken following the last data shown in Figure 3.18. One-phase exponential decay was also observed with black vinyl and hardwood carriers. The two-level exponential inactivation with tailing-off was
observed only with glass and loop-pile carpet materials, which exhibited the highest inactivation rates. Secondary exponential inactivation with lower rates followed this first phase.

3.4.6 Physical Effect of Chlorine Dioxide Exposure on Materials

All chlorine dioxide inactivation experiments were run for up to 5 hours. No apparent damage or discoloration of the carrier materials was observed during this period. No difference between before and after exposure to chlorine dioxide was detectable visually or through touch.

In order to assess any possible damage induced by chlorine dioxide, the same types of material used for ozonation experiments were exposed to 6 mg/L chlorine dioxide at 90% RH for longer periods of time. These materials were a calculator, floppy discs, compact discs, a zip disc, and several types of tygon and teflon tubings (Section 3.3.5).

The first failed device was floppy discs during the long term exposure to chlorine dioxide, as it was the case with ozone exposure. After 16 hours of exposure, some rust formations occurred at the disc head due to oxidation, nevertheless the disc did not fail. The disc failure occurred only after 18-20 hours exposure due to complete coverage of disc heads with rust. The zip disc failed after 25-27 hours of exposure,
but there was no rust formation over the disc head. Other materials continued to be exposed to chlorine dioxide for up to 40 hours. The calculator and the compact discs were not affected during this period. After the exposure they were continued to work properly.

There was no apparent change in teflon tubings after 40 hours chlorine dioxide exposure. Tygon tubings started to turn greenish yellow with time but when they were left in open air after the exposure, their color returned to their original colorless form. There was no apparent change in material characteristics. Plastics are known to absorb and desorb chlorine dioxide (86). During exposure, tygon tubings obviously absorbed chlorine dioxide, turning greenish yellow – the color of chlorine dioxide - while in open air they desorbed it back.

3.5 AQUEOUS INACTIVATION STUDIES

Inactivation of spores in aqueous phase with ozone and chlorine dioxide was conducted in phosphate buffered distilled water in the batch mode at pH 7.0 and 22°C with continuous stirring. In this chapter, the results of these experiments are presented.
3.5.1 Aqueous Phase Ozone Inactivation

The initial ozone concentrations used for the experiments ranged between 0.8 to 2.1 mg/L. The results presented in Figure 3.19 show that ozone concentration decreased rapidly with time. The area under the concentration curves was used to determine the CT value for each data point. The inactivation curves were plotted on Figure 3.20 with respect to time and CT. The stock ozone solution was added only at the beginning of the experiment, except for the run with 0.8 mg/L initial concentration, where additional stock ozone solutions were added intermittently to keep ozone concentration measurable throughout the experiment. The inactivation rate of spores and the CT values were adjusted accordingly.

![Figure 3.19 The change in ozone concentration in water during the course of experiment at pH 7.0 and 22°C.](image-url)
Figure 3.20 The inactivation of *B. subtilis* sp. spores in water phase with ozone at pH 7.0 and 22°C.
The CT concept was valid within the tested concentration range, as there was no significant difference observed among the experimental runs. All data points followed one single inactivation curve as shown in Figure 3.20. An initial lag phase was observed before the occurrence of exponential inactivation. This lag phase lasted for about 5.5 mg/L.min CT value. It was followed by a very fast inactivation of spores with one-log inactivation requiring 2.2 mg/L.min of CT. Overall, 5-log reduction was observed with just 16 mg/L.min of CT.

3.5.2 Aqueous Phase Chlorine Dioxide Inactivation

The initial chlorine dioxide concentration used for the experiments ranged between 4.5 to 7.1 mg/L. Chlorine dioxide concentration in water decreased slowly with time (Figure 3.21). The area under the concentration curves was used to determine the CT value for each data point. The inactivation curves were plotted on Figure 3.22 with respect to time and CT.

The CT concept was also valid for chlorine dioxide within the tested concentration range as there was no significant difference between the experimental runs. The inactivation curve obtained with chlorine dioxide was very similar in shape to the one obtained with ozone but the rate obtained was much slower. The initial lag phase lasted for about 40 mg/L.min CT value. The subsequent exponential inactivation phase required 45 mg/L.min CT per one-log reduction in the number of spores. Overall, 4-log reduction was observed with about 205 mg/L.min of CT.
Figure 3.21 The change in chlorine dioxide concentration in water during the course of experiment at pH 7.0 and 22°C.

Aqueous phase and gaseous phase inactivation data are shown on the same graph in Figure 3.23 for both ozone and chlorine dioxide for comparison of the inactivation in two phases.
Figure 3.22 The inactivation of *B. subtilis* sp. spores in water phase with chlorine dioxide at pH 7.0 and 22°C.
Figure 3.23 Comparison of the inactivation curves of *B. subtilis* spores obtained in aqueous phase and gas phase with (a) ozone and (b) chlorine dioxide at 22°C.
3.6 EFFECT OF GROWTH AND SPORULATION CONDITIONS ON INACTIVATION BY OZONE

The secondary objective of this study was to investigate the effect of several growth and sporulation conditions on the resistance of spores to inactivation. The procedure involved changing one or more components of growth conditions and examining the change in the resistance of spores to ozone inactivation. Chlorine dioxide gas was not used for this part of the study. Ozone was chosen as the model disinfectant due to its slower inactivation rate than chlorine dioxide. The results of these experiments are presented in this section.

3.6.1 Growth Solutions

Spores grown in two different liquid broth media, Nutrient broth (NB) and Tryptic soy broth (TSB), and on one solid agar medium, Tryptic soy agar (TSA), were used to study the effect of different growth media. This study was focused mainly on changing the concentration of cations in these growth media because DPA and peptidoglycan contents of the spores require positively charged cations for electron balance (Section 1.2.3). The main cations in the Schaeffer medium are Ca\(^{++}\), Mg\(^{++}\), Mn\(^{++}\), and Fe\(^{++}\) ions. Different growth solutions were obtained by changing the amount of these nutrients in the medium. The growth solutions used for this study are as follows: NB and TSB media with full Schaeffer (111) nutrients (NB-Schaeffer and TSB-Schaeffer), NB and TSB media without Schaeffer nutrients (NB-only and TSB-
only), TSB medium containing only one of the cations and no others (Ca-only, Mg-only, Mn-only, and Fe-only), and TSB medium with full Schaeffer nutrients plus ten times the concentration of one of the cations (Ca-plus, Mg-plus, Mn-plus, Fe-plus). It should be noted that broth and potassium-phosphate buffer were present in each solution. The spore populations in each solution are listed on Table 3.8.

The stock solution for TSA was obtained by collecting colonies formed over the agar surface. The final concentration of the TSA stock solution was brought down to \(2 \times 10^8\) c.f.u./mL to have similar number with the TSB solution.

### 3.6.2 Effect of the Growth Medium Type

The bacteria in NB with full Schaeffer nutrients had higher growth than in TSB with full Schaeffer. The bacterial growth was very limited in the absence of nutrients. The number of spores decreased by more than one-order of magnitude in both NB-only and TSB-only media in comparison to the NB-Schaeffer and TSB-Schaeffer media, respectively.

The spores from each solution were dried on glass strips and exposed to 10 mg/L ozone at 90% RH. The inactivation curves obtained with these spore solutions are shown in Figure 3.24. The inactivation curve of the S1 stock solution used throughout the ozonation experiments is also included for comparison.
Table 3.8 The number of spores in each test stock solution.

<table>
<thead>
<tr>
<th>Spore Solution</th>
<th>c.f.u./mL</th>
<th>Spore Solution</th>
<th>c.f.u./mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSB-Schaeffer</td>
<td>1.6*10^8</td>
<td>TSB-only</td>
<td>6.0*10^6</td>
</tr>
<tr>
<td>NB-Schaeffer</td>
<td>9.7*10^8</td>
<td>NB-only</td>
<td>7.2*10^7</td>
</tr>
<tr>
<td>Ca-plus</td>
<td>3.0*10^7</td>
<td>Ca-only</td>
<td>8.0*10^5</td>
</tr>
<tr>
<td>Mn-plus</td>
<td>2.2*10^8</td>
<td>Mn-only</td>
<td>8.4*10^6</td>
</tr>
<tr>
<td>Fe-plus</td>
<td>1.7*10^6</td>
<td>Fe-only</td>
<td>1.4*10^6</td>
</tr>
<tr>
<td>Mg-plus</td>
<td>1.1*10^8</td>
<td>Mg-only</td>
<td>1.1*10^7</td>
</tr>
<tr>
<td>TSA</td>
<td>2.0*10^8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.24 Effect of different growth media on inactivation of *B.subtilis* sp. spores with 10 mg/L ozone at 90% RH and 22°C.
The highest resistance to inactivation was observed with spores grown on agar surfaces, most probably due to the reaction of ozone with the solid particles attached to the spore colonies from agar surfaces. The spores grown without Schaeffer growth nutrients showed very little resistance to ozone. About 5-log inactivation was observed in 60 minutes with spores grown in NB-only and TSB-only whereas it was less than 3-log inactivation with spores grown in NB-Schaeffer and TSB-Schaeffer.

3.6.3 Effect of the Presence of Main Cations in the Growth Medium

In terms of the growth effect, presence of extra manganese (Mn-plus) and magnesium (Mg-plus) did not affect the final results, while extra calcium (Ca-plus) and iron (Fe-plus) resulted in less spore population. The extra iron (II) placed in the medium was mostly oxidized to iron (III). Settled iron (III) particulates formed at the bottom of container. The highest number of organisms grown in solutions containing only one cation and missing all others was at least one-order of magnitude less than those obtained in TSB-Schaeffer medium. The presence of only manganese (Mn-only) or magnesium (Mg-only) resulted in slight increase in total number of organisms in comparison to TSB-only, while presence of calcium (Ca-only) or iron (Fe-only) only had negative effect on total number of organisms. It should be noted that although other cations are missing from the growth medium, broth and potassium-phosphate buffer was still present in each solution.
The spores from each solution were dried on glass strips and exposed to 10 mg/L ozone at 90% RH. The inactivation curves obtained from these experiments are shown on Figure 3.25. The presence of extra calcium, manganese or magnesium resulted in only slight changes in inactivation curves compared to TSB-Schaeffer medium. Extra calcium (Ca-plus) caused in extension of lag phase, while extra magnesium (Mg-plus) resulted in higher inactivation rate for the first phase of inactivation. Inactivation curve obtained with extra manganese (Mn-plus) was not significantly different from the one obtained with TSB-Schaeffer. The biggest differences in spore resistance were observed with the absence of the cations and the presence of only one cation. The resistance obtained with the spores grown in the media with cations was significantly higher than the resistance obtained with spores grown in TSB-only solution (Figure 3.25b). The resistance of spores in Mn-only and Mg-only solutions was even higher than the spores grown in full Schaeffer medium or the media with extra presence of the cations. The spores in Fe-only solution had similar resistance to spores in Schaeffer medium while those in Ca-only solution had slightly less resistance.

3.6.4 Effect of Desiccation Time on the Resistance of Spores

The test strips prepared by drying spores on different carriers were kept in desiccators prior to exposure to gas inactivation for at least three days for all inactivation experiments. In order to check the effect of long drying times on the
Figure 3.25 Effect of presence of different cations in the growth medium on inactivation of *B. subtilis* sp. spores with 10 mg/L ozone at 90% RH and 22°C.
resistance of spores, additional inactivation experiments were carried out with glass carriers desiccated for two weeks and four weeks at less than 1% RH. Experiments were carried out with both ozone and chlorine dioxide gases. The inactivation curves obtained with spores desiccated for different times found to be not significantly different from each other (data not shown).

3.7 SUMMARY

The results presented on ozonation experiments demonstrate that gaseous ozone can be very effective against *B. subtilis* sp. spores. Ozone at 3 mg/L produced about 3-log reduction within 4 hours at 90% RH and 22°C on glass surface. No additional benefit was observed in terms of increased inactivation rate at higher ozone concentrations. The presence of relative humidity of more than 70% was essential for a feasible inactivation with ozone. Higher humidity levels during ozonation as well as pre-hydration of the spores increased the rate of inactivation. It was observed that the type of surface on which the spores rest either decreased or increased the inactivation rate of the spores significantly. Furthermore, different inactivation curves were obtained for different stock spore solutions which had different spore population and spore density.

Chlorine dioxide was observed to be very effective against spores of *B. subtilis* sp. spores. It was found to be a much stronger disinfectant than ozone in the gas form. Presence of more than 70% relative humidity was also essential for a feasible inactivation with chlorine dioxide. The inactivation process did obey the CT rule at
75% RH, but not at 90% RH. A CT level of 900 mg/L.min (5500 ppm.hour) was required to reach 6-log inactivation at 75% RH. Maintaining higher relative humidity decreased the total CT requirement. The type of the carrier materials affected the rate of inactivation, while the highest inactivation was observed over glass carriers.

The CT concept was valid in aqueous phase inactivation with both disinfectants. The inactivation curve obtained with chlorine dioxide was very similar in shape to the one obtained with ozone but the rate obtained was much slower. Tailing-off phase was not observed in aqueous phase inactivation with either disinfectant.

The results of the tests designed to investigate the effects of the growth and sporulation conditions showed that obtaining a consistent spore stock solution was very difficult. Although the type of the organic medium did not affect the inactivation rate, the spores grown in medium without the main cations showed very little resistance to ozone. This indicates the necessity of presence of the main cations during the spore formation. The addition of higher concentrations of the cations to the Schaeffer medium did not produce any significant changes. However, the presence of only one-cation caused increased spore resistance compared to the absence of the cation. Higher spore resistance was observed for spores grown in the presence of only magnesium or only manganese indicating the importance of these two cations to create resistant structures for spores. It was also observed that extended duration of the spore drying time did not affect the inactivation rate of spores.
CHAPTER 4

DISCUSSION

4.1 INTRODUCTION

This chapter provides a discussion of the experimental results that are presented in Chapter 3. Overall, the inactivation studies conducted with ozone and chlorine dioxide had many similarities in terms of the factors that affected the rate and the shape of the inactivation curves. Ozone and chlorine dioxide both produced a 2 or 3-stage inactivation curves. It is important to understand the significance of the shapes of the inactivation curves before making an attempt to analyze the factors affecting the inactivation process. Hence, this chapter was arranged first to discuss the shapes of the inactivation curves, which is then followed by a discussion of the results on the conditions affecting gas and aqueous phase inactivation.

4.2 INACTIVATION CURVES

The inactivation curves obtained during this study exhibited primarily three phases: First, a lag period or a shoulder during which no significant inactivation occurred. This phase was followed by the second phase, during which the
number of spores decreased according to first-order kinetics. The third phase involved “tailing-off” where the exponential decay in the number of spores continued, but the rate was reduced significantly. Not all experiments exhibited all these three phases. In gaseous chlorine dioxide studies, the lag period was absent; while in some other experiments the tailing-off was not observed at all. In addition, while the tailing-off was not observed in the aqueous phase studies with ozone and chlorine dioxide, the lag period was present in both aqueous systems.

4.2.1 The Lag Period

The initial lag phase can perhaps be explained by the oxidant demand of the organic constituents of the spore coat, and the time requirement of disinfectant to diffuse through the spore’s outer structures before the inactivation can occur. In Section 1.2.4, I suggested that the inactivation by ozone and chlorine dioxide might occur at the core membrane. To reach the membrane, the disinfectant should first diffuse through the gas film around the spore and then through the spore coat and the cortex.

The thickness of spore coat and cortex is known to change between 200 to 300 nm. The gas film thickness is also expected to be in nanometers range. The diffusion coefficients through these layers are not known. Therefore, the time required for diffusion of the disinfectant gas through these layers cannot be estimated. However, the time required for diffusion of water vapor through the spore coat and the cortex
was reported in an earlier study to be about 50 seconds (41). Using the Graham’s Law of Diffusion and assuming similar spore structures for the two studies, a diffusion time can be estimated for ozone and chlorine dioxide gases. The Graham’s Law states that “the rate at which gases diffuse is inversely proportional to the square root of their densities, which is directly proportional to their molar masses:

\[
Rate_{\text{diffusion}} \propto \alpha \frac{1}{\sqrt{\text{density}}} \propto \frac{1}{\sqrt{\text{Molecular weight}}} \tag{4.1}
\]

Taking the square root of the molecular weight ratio of ozone to water and chlorine dioxide to water, 1.6 and 1.9 values can be estimated for ozone and chlorine dioxide, respectively. Multiplying these values with 50 seconds, 80 seconds and 95 seconds diffusion times are estimated for ozone and chlorine dioxide, respectively. These time estimations do not include the time of diffusion through the gas film and time of reaction. These values were used in the following sub-sections for comparison of the lag phase durations observed in this study.

**4.2.1.1 The Lag Period in Gas Phase**

The lag period was observed only with ozone in the gas phase. The inactivation curves obtained for gas phase chlorine dioxide inactivation experiments did not show a lag period within the initial sampling interval of 5 minutes. Chlorine dioxide is not expected to react with either the coat or the cortex. Therefore, chlorine
dioxide should penetrate through the spore coat and the cortex without being consumed. In the earlier section, the diffusion time for chlorine dioxide was estimated to be about 95 seconds (1.6 minutes). During the experiments with chlorine dioxide, the first sample was taken at 5 minutes after the experiments were started. Therefore, we might conclude that even though a lag period might have occurred, it was probably missed because of our longer sampling intervals.

The length of the lag period observed with ozone gas varied between 5 to 20 minutes. The lag period increased with decreasing gas concentration and relative humidity (Figures 3.5 and 3.7). These values were much higher than the 80 seconds (1.3 minutes) estimated time for free diffusion. However, ozone has a high reactivity with proteins, and the spore coat contains high protein content. Hence, proteins at the spore coat can react with ozone, reducing the amount of ozone that may reach the spore core. Ozone consumption by these reactions may result in longer periods for an effective ozone concentration to reach the spore membrane. This may explain the delay in the inactivation, which was observed in the form of a long lag period. As ozone concentration is increased, more ozone will diffuse through the layers and reach the spore membrane, resulting in reduced lag times, as observed at high ozone concentrations.
4.2.1.2 The Lag Period in Aqueous Phase

A lag period was observed with both ozone and chlorine dioxide in the aqueous phase. The length of the lag period was 3-6 minutes with ozone (Figure 3.20) and about 7 minutes with chlorine dioxide (Figure 3.22). These values are significantly different than those observed in the gas phase. These differences can perhaps be explained by different diffusion and reaction rates in the aqueous and gaseous phases.

In the aqueous phase, the pores of the spore coat and the cortex is expected to be filled with water since water can move freely within these structures. Therefore, in the aqueous phase the disinfectant would diffuse not only through the liquid film around the spores, but also through the water-filled spore structures. The diffusion coefficients of ozone and chlorine dioxide are much lower in water than in air, as shown in Table 4.1. Hence, diffusion through water will take four-orders-of-magnitude longer time than diffusion through air for the same travel distance.

The lag period observed for ozone in water was lower than that observed in the gaseous phase. The observation that ozone was more effective in water than in the gaseous phase indicates the predominance of hydroxyl radicals (indirect mechanism) over ozone molecule in inactivation of the spores in water. As ozone diffuses through the watery spore structures, it will react and decompose into hydroxyl free radicals. Being indiscriminant oxidants, hydroxyl radicals will react immediately with
whatever layer they encounter, including the spore coat proteins and cortex peptidoglycan polymers and carbohydrates. Hence, the hydroxyl free radicals seem to attack and damage the coat and the cortex, rather than the spore membrane. This obviously will lead to faster inactivation compared to the mechanism where ozone or chlorine dioxide molecules will diffuse through the spore layers to reach the membrane for inactivation.

Chlorine dioxide is expected to inactivate the spores by the same mechanism in both the gaseous and the aqueous phases. The comparison of the observed inactivation rates in both phases supports this expectation (Fig. 3.23). The difference between the lag times of the two phases might be explained by different rate of diffusion of chlorine dioxide in the gaseous and aqueous phases. In the gas phase, the lag period, which was estimated to be about 1.6 minutes, was not observed within the initial sampling period. In the aqueous phase, the chlorine dioxide will diffuse through the liquid film, the spore coat and the water-filled cortex to reach the core membrane. This will take longer, as it was observed to be about 7 minutes during our experiments.

<table>
<thead>
<tr>
<th>Table 4.1 Diffusion coefficients of ozone and chlorine dioxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion Coefficient (cm$^2$/sec)</td>
</tr>
<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Air</td>
</tr>
<tr>
<td>Water</td>
</tr>
</tbody>
</table>
4.2.2 The Exponential Inactivation Phase

Following the lag phase, a logarithmic decrease in the number of spores was observed where inactivation followed pseudo-first order kinetics, as stated by the Chick-Watson Law (Eqn. 1.2). This phase was observed during all experiments. During this phase, 90% of the spores were inactivated within one D-value time. Within the subsequent one D-value time, 90% of the remaining spores, or 9% of the initial spores were inactivated. This behavior would continue until all of the organisms remaining in the medium are inactivated if the rate does not change. However, a decrease in the rate of inactivation with tailing off was observed during the experiments in the gaseous phase.

4.2.3 The Tailing-off Phase

The tailing-off phase was observed during the majority of the experiments, except in the aqueous phase. Tailing off has been frequently reported in the literature for inactivation of bacteria, viruses and spores in water. Several hypotheses regarding this behavior were proposed (95) to explain this phenomenon. Some researchers consider this observation to be due to an experimental error, while others attribute it to non-homogeneity of bacterial population or to the clumping of organisms. It was also suggested that the number of organisms would get too few after extended exposure times. As a result, the measurement technique which relies on statistically
random distributions may no longer apply (95). In this section, our results will be discussed in view of the hypotheses presented in the literature.

4.2.3.1 Small Population and Experimental Error

A small turn at the end of the inactivation curves could be understandable when only a few viable organisms remain in the medium. The measurement inefficiencies or some other effects may lead to high counts at the end points showing as the tailing-off of the data. However, in our studies, the tailing-off occurred well before the numbers of organisms got very small. For example, during ozone studies, tailing-off was observed after only 1-log inactivation. Therefore, it can be concluded that the experimental errors or small population could not have explained the observed tailing-off.

4.2.3.2 Non-homogeneity of Population

The tailing-off can also be explained by the presence of two or more different types of the same organism with different resistances against the disinfectant. In this case, the less resistant type of the organisms would have a higher inactivation rate exhibited by the first stage. When this stage of inactivation nears the completion, the more resistant population will start to dominate. The inactivation rate of this group of organism will be slower, causing the phenomenon of tailing-off. If two different populations exist in the stock solution, the ratio of their size is expected to determine
the tailing-off point in each inactivation curve. Furthermore, the same inflection point should be observed in each experiment conducted by the same culture. However, in this study the occurrence of the tailing-off changed from experiment to experiment. For example, during the ozonation studies, the tailing-off was observed after the occurrence of 0.9 to 1.5-log inactivation, depending upon the ozone concentration and humidity (Figures 3.5 and 3.7). In other words, about 3 to 13% of the initial population was still viable when the inactivation rate started to decrease. This point was at 1.8-log inactivation for the pre-conditioned samples (Figure 3.8). On paper and vinyl carrier materials, the tailing-off started to occur after 1-log inactivation. However, on the loop-pile carpet it was observed to occur after 2.3-log (0.5% viable) inactivation (Figure 3.9). On cut-pile carpet and hardwood, tailing-off was not observed at all although the experiments were continued up to 2.3-log and 1.7-log inactivation, respectively. The variation of the inflection point from experiment to experiment was even larger for chlorine dioxide. The tailing-off occurrence point varied between 0.9 to 4.5-log inactivation on glass carrier (Figures 3.13, 3.15, and 3.17). Yet on other materials the tailing-off was not observed at all with chlorine dioxide (Figure 3.18). The tailing-off did not occur during the aqueous phase studies with neither disinfectant (Figures 3.20 and 3.22).

These observations showed that even though the same spore solution was used for all the experiments, the tailing-off occurred at different inactivation points. These results suggested that this observation could not be explained by the presence of different species with different resistances to the disinfectants.
4.2.3.3 Clump Formation

The most probable reason for the tailing-off occurrence seems to be the formation of clumps between the spores themselves, and the clumps between the spores and the micro-particles remaining in the solution. Since the contact area is limited during gas inactivation studies, these clump formations may change the rate of inactivation considerably. The spores placed on the strips will have only the top surface area in direct contact with the disinfectant. During preparation of the test strips, clumping of spores may occur as mixed with micro-particles forming several layers on the test strips. Since the disinfectant gas will have to react and/or diffuse through the top layers before reaching the spores at the lower layers, the rate of spore inactivation will slow down causing the appearance of tailing-off. Therefore, the amount of clumpings was expected to change the rate of penetration as well as the rate of the inactivation of the organisms.

In order to evaluate the possible extent of clump formation, a simple calculation was performed based on the following assumptions: The mean size of the *B. subtilis* spores was reported to be 1.2μm long and 0.8μm wide under dry conditions (37). Therefore, on an average a spore might cover around 1μm² surface on the test strips. About 50μL of spore solution was placed on each test strips, and it was distributed over an area about 1cm by 3cm. This means that about 10⁸ c.f.u. of spores from the S2 solution were placed over the 3cm² area on the test strip. If the spores were to be taken individually and placed over the test strip, the area available per
spore would be only 3µm$^2$. However, it would be very difficult to place spores on test strips on a single layer. In addition, the spores tend to stick together, making it difficult to break them even in a liquid medium with high degree of stirring. Warriner et al. (116) observed the formation of spore clumps by a SEM on the surfaces of packaging board materials, even though only about $10^6$ c.f.u. of B.subtilis spores were placed over a 4cm$^2$ of surface area. The numbers of spores used in our study (S1 and S2 solutions) were about 4 and 100 times the number used by Warriner et al. Furthermore, it was likely that a large amount of micro-particles were mixed with the spores. Therefore, it is safe to assume that the formation of considerable amount of spore-clumping over the surfaces of the test strips was most likely the reason of the tailing-off. A similar situation was reported by Hury et al. (117), who studied the effectiveness of oxygen-based plasmas for inactivating spores on stainless steel. They observed a decrease in inactivation effectiveness with increased density of Bacillus spores.

With chlorine dioxide inactivation, no tailing off was observed on any material tested. However with ozone, the tailing-off either started later or did not occur at all on the carrier materials with rough surfaces i.e., carpet and hardwood materials, compared to glass. Rough surfaces typically present larger contact area, and as a result lower probability for clumping. In addition, the disinfectant gases can diffuse to reach the spores from underneath. These would lead to longer periods of first-phase inactivation, and consequently late or no occurrence of tailing off.
The dilution of spore solution resulted in higher first-phase inactivation rate (Figure 3.11). In addition, a smoother passage to the second-phase inactivation was observed with reduced difference between the rates of the first exponential phase and the tailing-off. For the highest dilution (S2-Dil 2), the rate difference was very small, suggesting a relationship between the density of the stock spore solution and the tailing-off occurrence. This further supports our conviction that clumping is the major reason for tailing-off since the clump formation is expected to decrease with increasing dilution of the spore solution.

In the aqueous phase studies, the spores were suspended in the medium under continuous stirring. Therefore, the extent of clumping of spores in water is expected to be much less in comparison to the dried test strips. Furthermore, the area available for the transfer of the disinfectant to the individual spores or the clumps would be the entire contact area between the spores and the bulk liquid, thus allowing the disinfectant to diffuse from all directions. Therefore, less tailing-off would be expected in water than in gas. This was confirmed by the results of aqueous and gaseous phase studies that are presented in Figure 3.23.

4.2.3.4 Effect of Gas Concentration and Relative Humidity on Tailing-off

As discussed previously, the inflection point between the first and second (tailing-off) exponential decay changed under different experimental conditions. In general, the inflection point was delayed with increasing disinfectant concentration as
well as increasing humidity, as shown in Table 4.2. Increasing the disinfectant concentration would increase the rate of diffusion through the spore structures proportionally. As a result, the disinfectant would reach the spores inside the clumpings faster and delay the start of the tailing-off phase.

The increase in the humidity level also delayed the inflection point. As it will be discussed in detail in section 4.3.2, the spores increase their size in response to an increase in the external humidity. The increase in the size of the spores might increase the spacing between them, and the spacing between the surrounding material and the other spores, thus decreasing the extent of clumping. In addition, the pores of spore structures – coat, cortex, and core membrane – might get larger due to the increase in the spore size in response to an increase in RH. As a result, the diffusion rate of the gas through these structures would increase causing a delay in the occurrence of the inflection points.

Table 4.2 The inflection point (log inactivation) of tailing-off occurrence on glass carriers during gas phase inactivation studies.

<table>
<thead>
<tr>
<th>O₃ conc. (mg/L)</th>
<th>RH (%)</th>
<th>Inflection point (log)</th>
<th>ClO₂ Conc. (mg/L)</th>
<th>RH (%)</th>
<th>Inflection point (log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>0.9</td>
<td>1.5</td>
<td>90</td>
<td>4.2</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>1.2</td>
<td>3</td>
<td>90</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>1.5</td>
<td>6</td>
<td>90</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>1.3</td>
<td>1.5</td>
<td>75</td>
<td>0.8</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>0.9</td>
<td>3</td>
<td>75</td>
<td>1.9</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>1.0</td>
<td>6</td>
<td>75</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>1.3</td>
<td>6</td>
<td>65</td>
<td>0.9</td>
</tr>
<tr>
<td>10</td>
<td>95</td>
<td>1.0</td>
<td>6</td>
<td>75</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>95</td>
<td>1.0</td>
<td>6</td>
<td>90</td>
<td>3.5</td>
</tr>
</tbody>
</table>
4.3 GAS PHASE INACTIVATION STUDIES

Gas phase inactivation studies with ozone and chlorine dioxide were performed using two different stock spore solutions (S1 and S2). Therefore, direct comparison of the inactivation rates and times was not possible between chlorine dioxide and ozone for every experimental condition. However, at 90% humidity on glass strips, the effectiveness of ozone and chlorine dioxide were tested on both spore solutions at 10 mg/L of ozone and 6 mg/L of chlorine dioxide. The results are compared in Figure 4.1 and Table 4.3.

Even though the ozone concentration (10 mg/L, 5000 ppm) was higher than the chlorine dioxide concentration (6 mg/L, 2200 ppm), chlorine dioxide was much more effective than ozone in gas phase inactivation of \textit{B. subtilis} sp. spores, as seen in the Figure 4.1 and Table 4.3. The time required for 5-log inactivation of spores in S2 stock was about 40 minutes with chlorine dioxide and 360 minutes with ozone. The time difference was even larger with less dense spore solution of S1.

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Stock Solution</th>
<th>First stage D-value (min)</th>
<th>Second stage D-value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/L ozone</td>
<td>S1</td>
<td>47</td>
<td>98</td>
</tr>
<tr>
<td>6 mg/L chlorine dioxide</td>
<td>S1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>10 mg/L ozone</td>
<td>S2</td>
<td>126</td>
<td>53</td>
</tr>
<tr>
<td>6 mg/L chlorine dioxide</td>
<td>S2</td>
<td>3.5</td>
<td>16.5</td>
</tr>
</tbody>
</table>
Figure 4.1 Comparison of the inactivation curves of *B. subtilis* spores obtained in gas phase with 6 mg/L chlorine dioxide or 10 mg/L ozone at 90% RH and 22°C.

The inactivation studies conducted with ozone and chlorine dioxide had many similarities in terms of factors affecting the rate and the shape of the inactivation curves. The increase in the gas concentration and the relative humidity resulted in an increase in the inactivation rate with both disinfectants. The usage of different carrier materials also resulted in the change of inactivation rates with both disinfectants. In the following sections, the factors affecting the gaseous inactivation are discussed. The inactivation rates of spores with ozone and chlorine dioxide are compared under different experimental conditions. The comparisons are based on the trends in the inactivation rates and the effect of environmental factors on the shape of inactivation curves.
4.3.1 Effect of Gas Concentration on Inactivation

The inactivation rate of the spores increased with increasing concentrations of both ozone and chlorine dioxide. In this section, first the relation between the gas concentration and the inactivation rates will be examined. Then the results of our experiments will be compared with the data available in the literature.

4.3.1.1 Concentration-Inactivation Rate Relation

An increase in the gas concentration resulted in an increase in the inactivation rate of spores for both ozone and chlorine dioxide. However, the relation was not first-order with respect to the disinfectant concentration in all cases. In other words, the relation given in Eqn. 1.2 (Chick-Watson’s Law) frequently did not hold.

It should be noted that according to Eqn. 1.3, i.e., \( C^nT_p = \text{constant} \), \( n \) does not have to be equal to 1. It was observed that \( n \) was equal to 1 for ozone inactivation up to an ozone concentration of 3 mg/L. With chlorine dioxide, \( n \) was not equal to 1 within the concentration range of 1.5-6.0 mg/L and at 90% humidity. Yet, \( n \) was equal to 1 for the same concentration range but at 75% humidity level (Figure 3.16).

These results show that the CT concept (i.e., \( n=1 \)), which is widely used in aqueous phase disinfection studies, may also be valid in gas phase applications only under limited conditions. The following analysis is presented to explain why the first-order relation may not hold under certain conditions in gas phase applications.
The Chick-Watson’s Law, which is based entirely on empirical observations, frequently fails even in aqueous phase disinfection studies. The first-order relation between the rate of inactivation and the concentration of the disinfectant, i.e. \( n=1 \), is justifiable if the rate determining step in the inactivation process is the reaction of the disinfectant with a susceptible moiety of the cell or the spore. However, the inactivation process involves several mass transfer steps for the disinfectant before the reaction can occur. These steps include the diffusion of the disinfectant through the stagnant liquid or gas film around the spore, and diffusion through the various layers within the spore before reaching the inactivation site. If the rate of any of these diffusion steps is slower than the final inactivation reaction rate, then the overall rate of inactivation will be controlled, and thus can be expressed by the rate of this diffusion process.

In the gas phase applications, the rate of diffusion of the disinfectant through the gas film around the spore is expected to be much faster than the rate of diffusion of the disinfectant through the internal layers of the spore. Assuming that \( C_b \) and \( C_i \) represent respectively the bulk concentration of the disinfectant and the disinfectant concentration at the inactivation site, the flux \( (J, \text{mole/cm}^2\cdot\text{sec}) \) of disinfectant through the spore layers can be presented by the Fick’s Law:

\[
J = \frac{D}{\delta}(C_b - C_i) \]

(1.1)
Here, it is assumed that the diffusion through the various layers of the spore can be represented by \( D \) (cm\(^2\)/sec), and the overall path-length the disinfectant has to travel from the surface to the inactivation site is represented by \( \delta \) (cm).

If the rate of diffusion is the rate controlling step, then the rate of inactivation can be presented as follows:

\[
\frac{dN}{dt} = -k' \times J \times \left( \frac{A}{V} \right)
\]  

(4.2)

where \( k' \) is a constant, \( A \) is the overall surface area of the spores (cm\(^2\)), and \( N \) is the number of spores present in the volume represented by \( V \) (cm\(^3\)). Furthermore, \( A = a \times N \), where \( a \) is the surface area of each spore exposed to the disinfectant. It is obvious that a spore exposed to the disinfectant from every direction will have a larger area and accordingly higher inactivation rate than a spore rested on a glass strip having only partial exposure. Hence,

\[
\frac{dN}{dt} = -k \times N \times (C_i - C_f)
\]  

(4.3)

where, \( k = k' \times \frac{D}{\delta} \times \frac{a}{V} \)  

(4.4)

If the diffusion rate is slow and/or the reactivity of the disinfectant is high, the disinfectant reaching the inactivation site would be consumed immediately, leaving
the concentration at the inactivation site \( (C_i) \) negligible in comparison to \( C_b \). In that case Eqn. 4.3 can be simplified to the following form:

\[
\frac{dN}{dt} = -k \times N \times C_b
\]  
(4.5)

This will be in agreement with the Chick-Watson’s Law where \( n=1 \). Furthermore, the integration of Eqn 4.5 will yield

\[
\ln\left(\frac{N}{N_0}\right) = -k \times C_b \times T
\]  
(1.2)

which leads to the so-called the “CT” rule.

However, if the diffusion rate is high under favorable conditions, a concentration build-up may occur at the inactivation site causing \( C_i \) to reach a value greater than zero. In this case, the first-order relation between the concentration and the inactivation rate (Eqn. 4.5) will not hold and the Eqn. 4.3 should be used instead. In fact, the inclusion of “\( n \)” in Eqn. 1.3 might have occurred when deviations from the first-order relation were observed, even in the aqueous phase.

Under constant conditions, the relation between the bulk concentration and the concentration gradient across the layers may be simplified by a power equation:
However, if one of the system characteristics is changed, the value for n would also change. Therefore, the value for “n” would be case-specific, changing with the environmental conditions. Nevertheless, the integration of Eqns. 4.3 and 4.6 will yield

\[
\ln \left( \frac{N}{N_0} \right) = -k \times \left( C_b^n \times T \right)
\]

which will explain the deviations from the “CT” rule.

The observations with ozone and chlorine dioxide in this study can now be explained better with these formulations. Inactivation by chlorine dioxide did follow the CT rule at 75% RH, but not at 90% RH (see Figures 3.14 and 3.16). As it will be discussed in Section 4.3.2, the pore dimensions of the spores increase with increasing humidity. At higher humidity, the pores of spores might have got larger causing an increase in the diffusion rate to allow the build-up of chlorine dioxide concentration at the reaction site. However, at lower humidity, slower diffusion of chlorine dioxide might cause chlorine dioxide to be depleted at the inactivation site so that Eqn. 4.5 would be applicable.

As shown by Eqn. 4.6, deviation from the linear CT rule at 90% RH might follow some power relation between the concentration gradient and the bulk concentration. The data obtained at 90% RH at different concentrations of chlorine
dioxide (presented in Fig. 3.14) were manipulated to fit both the first and second phase inactivation curves to a single $C^n T$ relation. The statistical error minimization technique indicated that $n=0.5$ for this set of experimental conditions. The data that were re-plotted by taking “$n$” as 0.5 fit a single inactivation curve, as shown in Figure 4.2. The chlorine dioxide concentration build-up at the inactivation site under these conditions can be estimated by using Eqn. 4.6 as $C_i = 0.28 \text{ mg/L}$ when $C_b = 1.5 \text{ mg/L}$, $C_i = 1.27 \text{ mg/L}$ when $C_b = 3.0 \text{ mg/L}$, and $C_i = 3.56 \text{ mg/L}$ when $C_b = 6.0 \text{ mg/L}$.

Figure 4.2 Inactivation of *B. subtilis* spores versus $C^n T$ taking $n$ equal to 0.5 for gaseous chlorine dioxide at 90% RH at 22°C.
For ozone inactivation, the CT rule applied well even at 90% RH up to 3 mg/L ozone concentration, but failed for higher concentrations (Fig. 3.6). Ozone and chlorine dioxide are expected to diffuse at rates close to each other, except ozone - being more reactive - is expected to react with spore coat proteins. These reactions will diminish the availability of ozone at core membrane, i.e., the inactivation site. This can explain the observation of first-order behavior, i.e. the applicability of the CT rule. However at high ozone concentration, such as 10 mg/L, ozone might still reach the inactivation site at appreciable concentration levels, and that would explain the deviation from the first-order relation and the CT rule.

4.3.1.2 Comparison of Numerical Values with Earlier Studies

The D-values obtained in two earlier ozonation studies were considerably lower than the values obtained in this study. Ishizaki et al. (5) determined the D-values as “12-13 min” for different strains of B.subtilis spores at 90% RH and 3 mg/L ozone concentrations. However, they observed much longer lag phases, 50 to 65 minutes, under the same conditions. Currier et al. (6) used very high concentration of ozone (9,000 ppm or 18 mg/L) for inactivation of B.globigii spores. Their results showed either no lag phase or very short ones, and more than 4-log reduction in the number of spores within 30 minutes, thus resulting in D-values of less than 10 min. In our study, a D-value of about 47 min was observed for inactivation at 90% RH using 10 mg/L of ozone. Although no significant difference in activation rates was observed
in our study between 3 and 10 mg/L ozone concentrations, the excessive ozone used by Currier et al. can partially explain their observation of very fast inactivation rates.

Although the general inactivation trend was similar in these three studies, the reported inactivation rates were not close to each other. A similar situation is also obvious in the water disinfection studies as researchers reported significantly different survival rates for various species of B. subtilis spores in the presence of aqueous ozone. These differences in the inactivation kinetics might partially be due to the physiological differences among the spores, which may change with changes in the sporulation medium. The physiological differences include the thickness, composition, and/or density of the spore coat, cortex and core membrane. In addition, differences in spore solution densities and pre-conditioning of the test strips could also lead to different inactivation rates.

Jeng & Woodworth (21) reported a D-value of 4.4 min for inactivation of B. subtilis spores with 30 mg/L chlorine dioxide gas at 80% RH and 30°C. In the present study, at 22°C and 6 mg/L of chlorine dioxide, the D-values were 13.1 min at 75% RH and 3.5 min at 90% RH. These values are comparable to those reported by Jeng & Woodworth, considering potential physiological differences among the spores in these two studies. No other studies on the inactivation rates of spores with gaseous chlorine dioxide were encountered in the literature.
In our study, a CT value of about 900 mg/L.min was required to reach 6-log inactivation on glass strips with chlorine dioxide at 75% RH. This is equivalent to about 5,500 ppm.hour chlorine dioxide exposure. For anthrax remediation applications, 9,000 ppm.hour of exposure was reported without specification of total inactivation level of the spores. Considering the extra precaution and safety needs for full scale applications, the reported exposure CT value seems to be realistic.

**4.3.2 Effect of Relative Humidity on Inactivation**

The experiments with ozone and chlorine dioxide showed that presence of high relative humidity medium was very important for effective inactivation of bacterial spores. The increasing relative humidity resulted in significant increases in the inactivation rates (Figures 3.7 and 3.17).

The relative humidity effect was not specific to only ozone and chlorine dioxide. Humidity was reported to enhance the inactivation of spores also with other gaseous disinfectants such as ethylene dioxide (112) and formaldehyde (118). Therefore, the effect may be more related to spore physiology than gas reactivity.

Earlier studies showed higher water sorption by spores at higher humidity (119) and a dynamic change in spores’ sizes in parallel to the change in relative humidity of air (41). Spores of *B.thuringiensis* were reported to grow or shrink in size
in response to increase or decrease in humidity (41). These studies showed that spores respond and adapt to environmental conditions including the relative humidity of air. The pores of spore structures also enlarge due to the increase in the spore size. Diffusion of the disinfectant gas through these structures might increase due to larger pore size and contact area. Higher diffusion rate of the gas would lead to higher inactivation rate of the spores.

The change in RH resulted in more steep changes of inactivation rates with chlorine dioxide than ozone. The inactivation level obtained at 65% RH was lower than 2-log in 300 minutes, while the time required to reach the same inactivation level was less than 30 minutes at RH values of 75 and 90%. The results also showed that 65% RH was not enough for “effective” inactivation, while 75% RH was sufficient to have 6-log inactivation within 160 minutes with 6 mg/L chlorine dioxide. At 90% humidity, the same level of inactivation was observed to be within 55 minutes.

The humidity effect for ozone inactivation was also significant but not as substantial as for chlorine dioxide. The inactivation obtained at 70% RH by ozone was just over 1-log within 240 minutes. However, the inactivation level reached about 4-log at 95% RH within the same time exposure and with the same ozone concentration. Increasing the humidity from 80% to 95% decreased the time required to reach 2-log inactivation from 230 minutes to only 110 minutes, or by just fifty-two percent.
Ishizaki et al. (5) observed a similar relation between the relative humidity and the inactivation rate of *B. subtilis* spores with ozone. These researchers observed proportional increase in the inactivation rate with increasing RH within the range of 50-95% RH. Contrary to these observations, Currier et al. (6) obtained significantly less kill at relative humidity higher than 80-85% RH when they exposed spores of *B. globigii* to 18 mg/L of ozone. These researchers used clumped spores in their experiments. They observed large variations between the experimental runs in addition to reduced inactivation rate at high humidity levels. These observations were explained by capillary condensation that might have occurred between the spores at higher humidity levels, which was implied by a sharp increase in water absorption above 80% RH. It was stated that water trapped between the spores would provide a significant mass transport resistance to the diffusing gas, thus reducing the overall kill rate (6). The extent of clumping must have been very high in that study causing large variations and non-reproducibility in the data. For example, the data varied from very little inactivation to complete kill under the same conditions. Therefore, it can be concluded that the observation of decreasing inactivation with increasing humidity must be specific to this study with highly clumped spores. We believe that if the degree of clumping is not very high, the occurrence of condensation may not be significant enough to reduce the rate of gas diffusion. Therefore, even though some deviations from the CT concept may occur, further increase in RH may not affect the mass transport of the disinfectant adversely. Thus, an increase in humidification of the
spore surface might result in acceleration of inactivation rate as observed in this research.

The results of this study show that the presence of relative humidity is a prerequisite to effective inactivation process of bacterial spores with gaseous ozone and chlorine dioxide. An effective inactivation requires at least 70% to 75% humidity levels. Furthermore, high level humidity is more crucial for chlorine dioxide than ozone application.

4.3.3 Effect of Pre-hydration on Inactivation

Pre-hydration of spores prior to exposure to ozone gas eliminated the initial lag-period and increased the inactivation rate of the first-stage slightly. Yet, there was no need for a prolonged pre-hydration as no difference was observed between 3-hour and 24-hour pre-hydration periods. However, chlorine dioxide started inactivation of spores with the beginning of exposure without any lag period, indicating no need for pre-hydration of spores. Thus, exposing spores to humid medium prior to exposure to chlorine dioxide did not result in the enhancement of the inactivation process. These results indicated that pre-hydration may not be needed at all as long as sufficient humidity is present during application of the gaseous disinfectant.
4.3.4 Effect of Carrier Material Surface on Inactivation

Spores were exposed to ozone and chlorine dioxide on strips of material that are in common use in offices and residences, such as glass, carpet, office paper, vinyl floor material, and hardwood. Different inactivation results were obtained on different material leading to the conclusion that the type of the material the spores rest on may affect the inactivation rate due to the disinfectant gas reacting with the material (Figures 3.9 and 3.18). The inactivation rates observed with different test carriers are ranked in Table 4.4 in descending order. The rankings were based on the results of the ANOVA statistical analysis. The materials were ranked the same when the statistical analysis did not show significant differences in the rates. This table would be helpful in differentiating the carrier materials with the highest and lowest inactivation rates. The slowest rate was observed on the finished hardwood floor while the glass carriers yielded the highest or the second highest inactivation rate for both disinfectants.

<table>
<thead>
<tr>
<th>Carrier material</th>
<th>Ozone First stage</th>
<th>Ozone Second stage</th>
<th>Chlorine dioxide First stage</th>
<th>Chlorine dioxide Second stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Brown Vinyl</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Office paper</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Carpet-loop pile</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Carpet-cut pile</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Black vinyl</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Hardwood</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 4.4 Statistical ranking of inactivation rates on different test carriers at 90% RH and 22°C from the fastest (1) to the slowest (7) rate.
Two different samples of carpet and vinyl were tested for this study. The carpet samples showed different results with both ozone and chlorine dioxide. However, the two vinyl samples resulted in similar inactivation levels with ozone but different inactivation levels with chlorine dioxide. Both vinyl samples and paper showed similar inactivation rates with ozone. With chlorine dioxide, brown vinyl and paper showed similar inactivation rate, while black vinyl had a lower rate. The results showed that brown vinyl tile behaved more like the paper surface probably because it had a top print layer, and the material underlying the top layer did not play a role during inactivation. However, the results with the black vinyl tile might be more representative of the behavior of vinyl surfaces encountered in office and residential spaces.

Glass, paper and vinyl floor carriers all yielded in the same level of inactivation with ozone with some lag phase, but the finished hardwood and old cut pile carpet resulted in lower inactivation levels than the glass, paper and the vinyl samples. Furthermore, the new loop-pile carpet sample showed the highest inactivation level and without a lag-phase.

Some volatile organic compounds (VOCs) are known to be emitted from carpet and hardwood surfaces. For example, Molhave et al. (100) reported that several types of VOCs mainly in the form of terpenes, which are reactive to ozone, were emitted from indoor wood surfaces. When these reactions occur, ozone is
consumed in the reactions within the boundary layer over the surface before ozone reaches the spores on the surface. As a result, less ozone becomes available for inactivation of spores. On the other hand, these reactions may create certain by-products that may serve as additional inactivation agents against the spores. For example, Weschler (99) and Morrison and Nazaroff (102), who studied the interactions between ozone and various carpet samples at typical indoor ozone concentrations of 30-400 ppb (0.06-0.8 μg/L) reported the formation of a variety of VOCs, generally aldehydes including formaldehyde. Formaldehyde was frequently used as a gaseous disinfectant, and also shown to be effective against *B. subtilis* spores (118). In addition, reactions between ozone and unsaturated hydrocarbons are known to produce a variety of products, ranging from short-lived highly reactive free radicals to stable highly oxidized species such as hydrogen peroxide (103). Hydrogen peroxide as well as various free radicals are also known as effective disinfectants.

We performed additional experiments in order to test the hypothesis that reaction by-products of ozone with the loop pile carpet can, in fact, function as a disinfectant and inactivate the spores. More than 90% less live spores were recovered from the pre-ozonated carpet strips compared to the non-ozonated ones (Figure 3.10). This is equivalent to inactivation of spores by more than 1-log on pre-ozonated carpet strips. This 1-log reduction may be attributable to inactivation caused by fumigative secondary emissions resulting from the reaction of ozone with the carpet surface. In fact, the difference between the remaining spores on the loop pile carpet and the glass
strips at the end of 4 hours of ozonation was also 1-log (Figure 3.18). This result implied that the enhanced inactivation observed on the loop pile carpet samples was due to the secondary emissions resulting from the reactions between ozone and the carpet surface.

With chlorine dioxide, the highest inactivation rate of spores was observed on glass carriers. The use of other carrier materials resulted in significant decrease and delay in inactivation in comparison to glass. Interactions between those carriers and chlorine dioxide, e.g., adsorption or chemical reactions, seem to reduce chlorine dioxide available for disinfection. However, no enhanced disinfection was observed on loop pile-carpet, which was the case for ozone.

Chlorine dioxide is known to react with chemicals selectively, but the available information on the types of chemicals reactive with chlorine dioxide is limited. It was reported that chlorine dioxide could be absorbed and desorbed by some surfaces, such as plastics (86). Our experimental results indicated that chlorine dioxide would probably be reacting with all the materials tested in this research, with the exception of glass. Furthermore, there is no indication that any of the reaction products has had any disinfecting capabilities.

The shape of the inactivation curves during chlorine dioxide exposure changed for some of the materials tested. The inactivation curves with office paper
and cut pile carpet had about 10 minutes of lag period, which was not observed with other material. The hardwood and the black vinyl produced a one-phase exponential decay with the slowest inactivation rate. This might be due to strong reactions of the chemicals on these two surfaces with chlorine dioxide. The two-level exponential inactivation with tailing-off was observed only with glass and loop-pile carpet materials where the highest initial inactivation rates were observed. The shape of the inactivation curve obtained with chlorine dioxide on brown vinyl material was different from the others. A slower inactivation rate was observed within the first hour of exposure followed by a steeper exponential decay. The first part seemed to be a lag-phase combined with a certain level of inactivation. Interactions between chlorine dioxide and top layer surface might be extensive during the first hour of exposure resulting in limited inactivation of the spores. After this period, the inactivation rate increased substantially.

For this study, glass proved to be a good selection as a neutral base material causing no apparent reaction with ozone or chlorine dioxide. Yet, the rates observed on glass for both disinfectants were extreme overestimations of the realistic rates. For remediation of contaminated offices and residences the rates observed for hardwood surfaces should be adopted as a basis for the standard of practice.
4.3.5 Comparison of Physical Effect on Materials

No apparent damage or discoloration of the materials was observed after ozone or chlorine dioxide exposures during the experiments. There was no difference between the materials before and after the exposures that can be determined visually or through touch. In order to assess the possible damage induced by the disinfectants, several devices were exposed to ozone and chlorine dioxide at 90% RH for longer periods of time. These devices and materials included a calculator, floppy discs, compact discs, zip discs, and different types of tygon and teflon tubings. These simple devices were used as simulators of more complex electronic equipment.

The floppy discs, which are relatively sensitive and sometimes fail with no apparent reason after a few usages, were the first to fail to both ozone and chlorine dioxide. They failed after 9-11 hours of ozone and 18-20 hours of chlorine dioxide exposure. Then the zip discs failed after exposures of 20-23 hours with ozone and 25-27 hours with chlorine dioxide. The calculator was not affected by ozone exposure of 34 hours and chlorine dioxide exposure of 40 hours. There were no visible significant changes in the tubings after 40 hours of exposures.

These results showed that most of the devices and materials present in typical office or a facility would be safe over the time of the decontamination process, which should take less than 8-10 hours. However, paintings, antiques and other expensive
furniture should be removed to prevent any possible deterioration that may come from the disinfectant exposure.

4.4 AQUEOUS PHASE INACTIVATION

The inactivation curves obtained with ozone and chlorine dioxide in the aqueous phase are shown on the same graph in Figure 4.3. The shape of the inactivation curves including the duration of the lag period and observance of only one-phase inactivation without tailing-off was discussed in detail in Section 4.2.

The relation between the inactivation of the spores and the concentration of the disinfectants was first order for both disinfectants, i.e., the data followed the CT rule. This observation indicates that the disinfectant concentrations at inactivation sites ($C_i$) should be negligible in comparison to the bulk concentrations ($C_b$) of both disinfectants.

Ozone inactivation in the aqueous phase was very fast. Hydroxyl radicals produced in water, due to their extreme reactivity are never expected to accumulate at the inactivation site. Therefore, the inactivation rate is expected to be directly proportional to the bulk ozone concentration, as observed (Figure 4.3).
In the case of chlorine dioxide, the rate of diffusion of chlorine dioxide is obviously slower than its reaction at the inactivation sites so that the CT rule and Eqn. 4.5 were both applicable.

As seen from the Figure 4.3, the CT value required to reach a certain level of inactivation was much higher with chlorine dioxide than ozone. The CT required for the same level of inactivation was about 20 times less for ozone than that was required for chlorine dioxide. Therefore, ozone was shown to be a more effective disinfectant than chlorine dioxide in aqueous-phase inactivation of the spores.
The findings on ozone are in line with the results given in the literature for inactivation of spores with ozone. Several researchers studied the inactivation of *B. subtilis* sp. spores with ozone in the aqueous phase. All observed a lag phase followed by an exponential decay. The lag time and the inactivation rates differed from each other but overall 5-log inactivation was observed between 7 to 13 mg/L.min CT values under the experimental conditions similar to this study (18, 20, 92). Our results indicate a CT value of about 16 mg/L.min for 5-log reduction (Fig. 3.20). Considering the possible variations in spore preparation and experimental procedures, the findings of our study are comparable to those of the earlier studies.

Few publications appear in the literature on chlorine dioxide inactivation of spores in water. Radziminski *et al.* (94) studied the inactivation of *B. subtilis* spores by chlorine dioxide in the aqueous phase. Although chlorine dioxide exist in water in molecular form and its concentration is not expected to vary with pH, these researchers obtained different inactivation curves in laboratory water at different pH values (94). For example, the CT value required to obtain a 4-log of inactivation was about 210 mg/L.min at pH 6.0 and 80 mg/L.min at pH 8.0. Our results obtained at pH of 7.0, 22°C in phosphate buffered distilled water indicate a CT value of about 205 mg/L.min, and it is closer to the results reported for pH 6.0.

The results of this study show that aqueous phase and gas phase inactivation rates of the spores with ozone and chlorine dioxide vary substantially even for the
same spore solution. In following parts of this section, a comparison of aqueous phase and gas phase inactivation rates will be presented for both ozone and chlorine dioxide.

4.4.1 Comparison of Ozone Inactivation in Gas and Aqueous Phases

The CT rule was found applicable to the ozone data in the aqueous phase over the initial ozone concentration range of 0.8-2.1 mg/L (Fig. 3.20). The requirement for ozone to reach a certain level of inactivation was substantially faster in water than in gas (Figure 3.23). For example, while a CT value of only 11 mg/L.min was required to reach the 3-log inactivation in water (Figure 3.20), this value was 750 - 2,400 mg/L.min at 90% RH in the gas phase, depending upon the gas concentration (Figure 3.6). As explained previously, oxidation mechanism of ozone in water includes free radical formation, whereas primarily direct oxidation will occur in the gas phase. Hydroxyl free radicals produced in water might attack and damage the spore coat and the cortex, leading to a much faster inactivation compared to ozone molecule diffusing through the spore layers to reach the spore membrane to damage the spore.

In the aqueous phase, the first-order relation (CT rule) is expected to hold at all levels of ozone concentrations based on the analysis presented in Section 4.3.1.1. Hydroxyl radical, due to extreme reactivity is never expected to accumulate at the inactivation site, and hence $C_i$ will be negligible compared to $C_b$, the ozone bulk concentration, and therefore, Eqn. 4.5 will apply, as observed.
Table 4.5 Comparison of the CT values (as mg/L.min) obtained with chlorine dioxide in gaseous and aqueous phases at 22°C.

<table>
<thead>
<tr>
<th>Observed log inactivation</th>
<th>CT in gas at 90% RH</th>
<th>CT in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-log</td>
<td>12 – 21</td>
<td>70</td>
</tr>
<tr>
<td>2-log</td>
<td>24 – 42</td>
<td>115</td>
</tr>
<tr>
<td>3-log</td>
<td>36 – 63</td>
<td>160</td>
</tr>
<tr>
<td>4-log</td>
<td>48 – 138</td>
<td>205</td>
</tr>
</tbody>
</table>

4.4.2 Comparison of Chlorine Dioxide Inactivation in Gas and Aqueous Phases

The CT value observed for various inactivation levels are compared in Table 4.5 for chlorine dioxide in the gaseous and aqueous phases. In general, higher CT values were needed in water than in gas for the same level of inactivation. This could be attributable to slower diffusion of chlorine dioxide through the water film and the water-filled spore layers. The observation of n=1 for water, and n=0.5 for the gas phase also indicate slower diffusion in water preventing accumulation of chlorine dioxide at the inactivation sites. On the other hand, chlorine dioxide molecule is obviously responsible of the inactivation in both phases, as expected.

4.5 EFFECT OF GROWTH AND SPORULATION CONDITIONS ON INACTIVATION BY OZONE

The secondary objective of this study was to investigate the effect of several growth and sporulation conditions on the resistance of spores to inactivation. When the growth conditions of the bacteria were changed, the growth rate changed, resulting
in a different final population in the medium. The same initial medium volume was used for all experiments. Therefore, different spore population densities were obtained in each medium. Different stock solutions prepared under the same conditions resulted in different inactivation curves due to stock density differences as discussed in section 4.5.1. Therefore, the data were analyzed and discussed based on both the inactivation curves and the spore population differences in order to evaluate the effect of growth and sporulation conditions. Ozone was chosen as the model disinfectant due its slower inactivation rate than chlorine dioxide.

4.5.1 Effect of Stock Spore Solution on Inactivation Rate

A high population spore stock solution was prepared to study the effect of stock solution density on the inactivation rate. Higher population was obtained by using more growth solutions and through centrifugation. Two stock solution (S1 and S2) and dilutions of S2 were used as different stock solutions.

The slowest inactivation rate was observed with S2 stock that contained the highest spore density. The dilution of stock solution S2 with distilled water resulted in a higher inactivation rate. With S2-Dil 1 and S1 solutions, which contained about the same spore densities, similar first phase inactivation rates were obtained. The highest inactivation rates were observed with S2-Dil 2, as it had the lowest spore density. This phenomenon of dependence of the inactivation rate on initial spore density can be explained by more clump formation in more dense solution. Media particles that exist
in solutions together with the spores are expected to help with clump formations. Hence, in diluted solutions with less clumping and more readily available spore surface, disinfectants become effective in inactivating greater number of spores.

Even though all the solutions were prepared under similar conditions with the same start-up culture, the amount of growth solution used to prepare the stock solutions was different for each case because of different resulting spore populations. In order to have a higher density, more growth medium was prepared and concentrated. After the growth period, the solutions were centrifuged and washed several times. However, micro-particles formed during growth and spore formation possibly remained in the solution since it was not possible to differentiate the particles from the spores. The procedure of centrifugation concentrated both the spores and the particles. The high density of the solution and the presence of high amount of other particles have possibly increased the degree of clumping, resulting in slower inactivation rates. These results might explain the differences observed between different gas phase inactivation studies since it would be unlikely to obtain similar spore solutions in all studies.

4.5.2 Type of the Growth Medium

The presence of full Schaeffer nutrients had resulted in higher bacterial growth and as a result higher spore density, as expected. The number of spore population decreased by more than an order of magnitude in the absence of nutrients in both
nutrient broth (NB) and tryptic soy broth (TSB) media in comparison to the corresponding full Schaeffer nutrient media.

The spore population obtained in NB was higher than that obtained in TSB under the same conditions. Apparently, the NB solution was more favorable for the growth of \textit{B.subtilis} organisms. However, the statistical analysis showed no significant difference between the inactivation rates obtained with spores grown in NB and TSB.

The presence of all the necessary nutrients in the broth medium also made a significant difference on the resistance of spores to inactivation. The spores grown without Schaeffer nutrients had very little resistance to ozone. About 5-log inactivation was observed in 60 minutes with spores grown without main cations (TSB-only and NB-only) (Figure 3.24). However, with spores grown in full nutrient media (TSB- and NB-Schaeffer), only about 3-log inactivation was reached within the same time-exposure. The results showed the necessity of the presence of the cations not only for the growth of bacteria but also for the resistance of spores. The spore structure is known to need the cations mostly in the spore core and cortex to electro-balance DPA in the core and peptidoglycan in the cortex, which are very important elements of the spore resistance to external conditions.
The S1 and TSB-Schaeffer solutions had about the same spore densities, yet the inactivation curves were very different. The spores of TSB-Schaeffer showed less resistance to ozone than the spores of S1 solution (Figure 3.24). This observation can perhaps be explained by the different concentrations of particles, as reflected by turbidity. The S1 solutions had a much higher turbidity than the TSB-Schaeffer solutions. The S1 solution was prepared in large amounts in several reactors, while the solutions used for experiments with different nutrients were prepared in single container with low broth volume. The low volume solutions might have had better mixing conditions and aeration providing higher bacterial growth. Since the volumes used to grow S1 solutions were higher, less aeration and mixing applied to S1 might have resulted in less bacterial growth and sporulation. This, in turn, resulted in more centrifugal concentration of the medium to get the final stock spore solution population. Obviously, as more centrifugal concentration was applied, the denser the micro-particles got, causing less inactivation rate, as discussed earlier.

These results showed the difficulty of experimentally getting a consistent spore stock solution. In order to produce stock solutions with similar morphological and physical characteristics and similar spore population and overall particle density, not only the same growth medium, constituents and physical conditions should be provided, but also the same procedure should be applied for preparation of the solutions. Since the rate of inactivation by gaseous ozone showed dependence on the
all these factors, widely varying experimental results from different studies would be expected.

4.5.3 Effect of the Presence of Main Cations in the Growth Medium

The growth in all the solutions containing only one cation - while missing the others - were at least an order of magnitude lower than the growth obtained in the Schaeffer medium; this showed the necessity of the presence of all the cations to obtain a high growth rate (Table 3.8). The presence of only manganese or magnesium resulted in a slight increase in the growth rate in comparison to TSB-only, while the presence of only calcium or iron (II) produced a negative effect on the growth rate. It should be noted that although other cations were missing from the growth medium, the broth and potassium-phosphate buffer were present in each solution. The negative effect of calcium can be explained by formation of calcium-complexes with phosphate and other anions, which may no longer be available to the organisms.

The presence of extra calcium, manganese or magnesium has produced only slight changes in the inactivation rates compared to the Schaeffer medium (Figure 3.25). The resistance of spores grown in the presence of extra iron was much less than the resistance of others. As it was explained previously, extra iron mostly oxidized to iron (III) in the solution. Iron oxides may have inhibited the growth of bacteria and/or formation of spore structures. Extra calcium resulted in extension of the lag phase, while extra magnesium resulted in higher inactivation rate for the first phase of
inactivation. The inactivation curve obtained with extra manganese was not significantly different from the one obtained with the Schaeffer medium. Longer lag phase with extra calcium might be due to facilitation of clumpings of the spores by the calcium complexes. The inactivation rates of the secondary phases for each case were similar, except for the extra iron solution. In summary, the use of high amounts of cations did not result in significant changes in the spores’ resistance.

The largest difference in spore resistance was observed with the presence of only one of the cation and the absence of the others in the solution (Figure 3.25). The resistance of spores grown in manganese-only and magnesium-only solutions was higher than the resistance obtained in the Schaeffer medium. Calcium-only and iron-only solutions also produced comparable resistance to the Schaeffer medium. When only one of the cations is present in the medium, this cation might be used in the spore structures as a substitute of the other cations which, in turn, might increase the resistance of the spores. This may explain the results with manganese or magnesium when they were present as the only cation source. Therefore, it can be concluded that manganese and magnesium might be more important than calcium and iron for the bacterial growth and the formation of spore resistance structures.

4.5.4 Effect of Desiccation Time on the Resistance of Spores

Desiccation time of spores before exposure to ozone or chlorine dioxide did not result in different inactivation rates of the spores. The resistance of *B.subtilis*
spores kept under desiccation from 3 days to four weeks did not change significantly with change in desiccation time. As a result, it can be concluded that the spores kept under dry conditions for longer period of times might not lead to increased resistances to ozone and chlorine dioxide.

4.6 PRACTICAL CONSIDERATIONS

The procedure to be followed for decontamination of a facility would be the same for both chlorine dioxide and ozone gases, except for the gas generation and the destruction systems. The preparation of the facility, control and post-treatment procedures might be similar since both ozone and chlorine dioxide dosage requirements would be well above their toxic levels.

First, the facility should be isolated and sealed to minimize the escape of the gas to the surrounding atmosphere. The sunlight through the windows should be blocked to minimize the gas degradation. Gas and relative humidity monitoring systems should be placed throughout the decontamination area to ensure reaching the desired humidity and disinfectant levels everywhere and uniformly for the duration of the decontamination process. If the facility has a HVAC system, the gas might be pumped in and circulated through this system to distribute it uniformly throughout the facility. In the absence of an HVAC system, supply pipes and fans may need to be
placed throughout the facility. Steam may be introduced ahead of the disinfectant gas to increase and maintain the desired humidity level and temperature.

The chlorine dioxide generation systems typically use pre-blended chlorine-nitrogen feed from compressed-gas cylinders or tube trailers and solid sodium chlorite cartridges/reactors. The gas cylinders and sodium chlorite supplies can be brought to the decontamination site for on-site generation of chlorine dioxide. However, since the production involves the use of compressed toxic gas in large containers, handling and transportation require care to prevent serious accidents. In the case of ozone, generator(s) should be brought to the decontamination site for on-site ozone generation. Ozone generation systems need only a power supply and air compressor as they can have oxygen concentrators concentrating oxygen directly from air. This oxygen is then supplied into the ozone generator for ozone generation. Neither chlorine dioxide nor ozone generation systems require mixing or contact with any chemicals. The start-up and shut-down of the systems are very quick. However, in the case of chlorine dioxide, large amounts of the chlorine gas and chlorite needed at the site for generation of chlorine dioxide will most likely create hazardous conditions during their transportation as well as on-site storage since these chemicals are hazardous. Ozone, on the other hand, will not have this problem since ozone generators will be using air as the only raw material. This is obviously an advantage of ozone over chlorine dioxide.
After the decontamination process is completed, air can be pumped into the facility to dilute chlorine dioxide or ozone. The exhaust gas can be passed through a gas destruct device to safely release the gases into the atmosphere. Chlorine dioxide exhaust can be passed through sodium bisulfite to neutralize chlorine dioxide. Thermal-catalytic destruct modules can be used to destroy ozone in the exhaust. However, that may not be needed at all since ozone decomposes back to oxygen over time. As a result, although chlorine dioxide residual and odor will continue to be a nuisance for people who return to the remediated spaces, especially for those who may have allergic reactions to this odor, ozone will not linger around to cause such problems. This is obviously another advantage of ozone over chlorine dioxide.

In spite of the disadvantages of chlorine dioxide from the points of view of safety and potential subsequent health problems, it produces a faster inactivation rate of spores compared to ozone. Yet, the difference in these rates, e.g., 40 minutes of chlorine dioxide or 360 minutes of ozone application, may not be a significant benefit to the society compared to the safety and health risks associated with chlorine dioxide.
CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 SUMMARY AND CONCLUSIONS

This research was conducted to investigate the proper operational conditions for the gaseous disinfectants, ozone and chlorine dioxide, to effectively inactivate *Bacillus subtilis*, a spore forming non-pathogenic bacteria that belong to the genus *Bacillus*, and share the same physiological characteristics as *Bacillus anthracis* that cause the infectious anthrax disease. The gas-phase studies were conducted in specifically constructed ozone and chlorine dioxide gas chambers where constant gas concentrations were maintained by creating continuous-flow and completely-mixed conditions under steady-state. The experiments were conducted to investigate the effect of gas concentration, contact time, relative humidity, pre-hydration, carrier material, and growing and sporulation conditions. The aqueous phase studies were conducted at pH 7 and 22°C under batch conditions with varying concentrations of the disinfectants.

Both ozone and chlorine dioxide were observed to inactivate the spores effectively in both the gaseous and the aqueous phases. In the gaseous phase chlorine
dioxide was found to be much more effective against the spores than ozone under similar conditions. However, in the aqueous phase ozone was a more superior disinfectant than chlorine dioxide against the spores.

In the gas phase, the rate of inactivation was observed to be directly proportional to the gas concentrations (C) of ozone and chlorine dioxide. However, for ozone increasing the gas concentration beyond a certain point did not bring any additional benefit. The time of contact (T) was the second important factor affecting the extent of the inactivation. The rate of inactivation by ozone and chlorine followed the CT concept only under limited concentration and humidity levels. More frequently, the CT rule did not apply to the gaseous disinfection of the spores. A proposed disinfection model based on diffusion limitation of the disinfecting gas was successful in mechanistically explaining the reasons for deviations from the linear CT rule. This model considers the inactivation as a process involving diffusion of the disinfectant through a fluid film and layers of the spores followed by the reaction of the disinfectant at a vulnerable site in the spore membrane. If the diffusion rate of disinfecting chemical is faster than the reaction rate, a concentration build-up may occur at the inactivation site causing deviation from the linear CT rule.

The presence of relative humidity was essential for effective inactivation of the spores. The results showed that inactivation might not be practical at humidity levels below 70%. Increasing the relative humidity of the air above 70% resulted in an
increase in the rate of inactivation. Pre-hydration of the spores before inactivation was found to be unnecessary as long as sufficient humidity could be provided during inactivation.

Overall, chlorine dioxide was observed to be at least 10 times more effective than ozone. About 285 – 820 ppm.hour chlorine dioxide dosage was required to reach four-log inactivation level of *Bacillus subtilis* spores at 90% RH depending on chlorine dioxide concentration. Under the same conditions, the CT requirement for ozone was 9,700 ppm.hour when applied ozone concentration was 1-3 mg/L.

The experiments with different surface materials showed that the type of surface on which the spores rest could affect the inactivation rate of the spores significantly. The lowest inactivation rate of the spores was observed for finished hardwood test strip for both ozone and chlorine dioxide, indicating a demand by the chemicals of the hardwood material for ozone and chlorine dioxide. Considering the fact that most offices and residences usually have large surfaces of hardwood, the rates observed for hardwood surfaces should be adopted as the standard of practice for remediation of contaminated enclosed spaces.

No visible damage to the materials was observed as a result of exposure to ozone or chlorine dioxide. The items tested for durability were still functional after 20 hours of exposure to ozone or chlorine dioxide, with the exception of the floppy discs.
Therefore, materials that may be exposed to these disinfectants during remediation efforts are expected to be safe, although it is advisable to apply special care to antiques and artwork.

The results on the effect of growth and sporulation conditions of the spores indicated that the presence of the main growth nutrients was essential for the growth and the resistance of spores against ozone inactivation. However, extra nutrients did not provide any additional resistance. The presence of only one cation in the growth medium, with others being absent, produced spores that exhibited the same or higher resistance than the spores formed in full nutrient solution. The presence of several cations, especially magnesium and manganese, in the growth medium was required to create high resistance of the spores against inactivation.

In the aqueous phase, the inactivation data obtained for both ozone and chlorine dioxide followed the C.T rule, indicating that the disinfection was a diffusion-limited process for both disinfectants. The relatively lower inactivation rate in the aqueous phase than in the gaseous phase for chlorine dioxide can be attributed to a slower diffusion of chlorine dioxide through water-filled spore structure in the aqueous phase. However, the extremely fast inactivation rate by ozone in the aqueous phase compared to the slow rate observed in the gaseous phase can only be explained by the predominance of hydroxyl radicals over ozone molecule as the inactivating agents in the aqueous phase.
In conclusion, this research has provided the groundwork for application of gaseous ozone and chlorine dioxide for remediation of enclosed spaces contaminated with biological agents. The results on the effects of the operational conditions on the inactivation rate - including gas concentration, contact time, relative humidity, and the type of the carrier material – can be used for planning purposes in efforts involving remediation of contaminated facilities.

Furthermore, this research has provided valuable information on the inactivation patterns of *Bacillus subtilis* spores by ozone and chlorine dioxide in the gaseous and aqueous phases. The data indicate that the process of inactivation of the spores involves consecutive steps of diffusion of the disinfectants through the gas/liquid film around the spore and various layers of the spore, culminating by the reaction at a site reactive with ozone, chlorine dioxide or free radicals.

**5.2 RECOMMENDATIONS**

Different spore stock solutions prepared in this study produced different results for the rates of inactivation and growth even though similar experimental conditions and procedures were applied. For future studies, it is recommended that a protocol is created to obtain a single representative stock solution by applying strict uniform physical and chemical conditions so that different studies can produce comparable experimental results.
In this study, inactivation of spores was performed on different carrier materials, yet without an in-depth analysis of the surfaces before and after exposure to the disinfectants. It is recommended that the reactions between the surfaces and ozone and chlorine dioxide are studied in the future at a fundamental level to be able to better predict the effect of exposure to these chemicals.
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