University of Alberta

# EVALUATING THE DISINFECTION EFFICIENCY OF OZONE AND FREE CHLORINE FOR ATTACHED (BIOFILM) AND SUSPENDED BACTERIA IN NEW AND REPAIRED WATER MAINS

by

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## DEDICATION

I dedicate this thesis, and all the effort that went into it, to my wife, Shoshma, for her love, support, and inspiration from the start, through to the finish of this work, and beyond, and to my daughter, Sadhna, and son, Ashlesh, who always showed their presence with great smiles.

## ABSTRACT

Biofilm disinfection in new and repaired water mains is conventionally achieved by using chlorine. Considering the practical limitations of chlorine for biofilm disinfection, ozone was investigated as an alternate disinfectant. A number of laboratory experiments were carried out for biofilm disinfection with ozone and free chlorine at "field conditions" (i.e. different concentration-time or Ct products). The results were compared with those of suspended bacteria in terms of log inactivation of heterotrophic plate count bacteria.

The results show that biofilm inactivation was limited to 1 log for an ozone Ct value of up to 56 mg x min/L in comparison to over 3 log inactivation of suspended bacteria at Ct value of up to 62 mg x min/L. The bacterial inactivation was greater than the measurement limit for both biofilm and suspended bacteria at free chlorine Ct of over 40,000 mg x min/L. At these "field conditions", ozone was considerably less effective than free chlorine, particularly for biofilms.

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# CHAPTER 1: INTRODUCTION

## 1.1 Background/Overview

The occurrence of bacterial regrowth in potable water mains has been reported since the findings about the regrowth of *Escherichia coli* (United States Environmental Protection Agency or U.S. EPA, 1992; Percival et al., 2000). Further research in later years confirmed that, under favorable growth conditions, these bacteria can grow on the walls of potable water mains, in the form of biofilms (Prévost et al., 1998; Haas et al., 1999; Percival et al., 2000).

Despite accumulated evidence confirming the presence of biofilms on potable water mains, a biofilm-associated public health concern was not realized until late 1970s. It was shown that pathogens, opportunistic pathogens, viruses, and protozoa in potable water mains can grow into biofilms (Geldreich, 1996; Armon et al., 1997; Camper, 2000; Percival et al., 2000; van der Kooij, 2003). It is believed that some microorganisms survive the treatment processes (Geldreich, 1996) and can be introduced during contamination into the water distribution system via cross connections, water main breaks or pipe repairs and/or backsiphonage (Geldreich, 1996; Haas et al., 1999; Kirmeyer et al., 2001). These microorganisms may result in the formation of biofilms that can provide a shelter to disease-causing bacteria against adverse conditions (Camper, 1996 and 2000; Geldreich, 1996; Percival et al., 2000; van der Kooij, 2003).

There are a number of factors that can be responsible for the biofilm formation and growth in the potable water mains. These factors include the type and quality of source and treated water (LeChevallier et al., 1991; Prévost et al., 1998; Camper, 2000; Menaia and Mesquita, 2004), the effectiveness of disinfectant residual (LeChevallier et al., 1991; Prévost et al., 1998; Ollos et al., 2003; van der Kooij, 2003), the type and quality of water main materials (LeChevallier et al., 1991; Geldreich, 1996; Camper, 2000; Ollos et al., 2003; Menaia and Mesquita, 2004), system hydraulics (LeChevallier et al., 1988a; Menaia and Mesquita, 2004), water temperature, and rainfall events (LeChevallier et al., 1991; Geldreich, 1996; Camper, 2000; Ollos et al., 2003; Menaia and Mesquita, 2004).

The presence of biofilms in the potable water mains can be associated with many problems including turbidity, taste and odor, production of red or black waters, interference with detection of coliforms indicators, increase in frictional resistance of water main causing reduced water carrying capacity or loss of pressure, and non-compliance with treated water quality requirements (McFeters, 1990; Camper, 1996; Camper, 2000; Prévost et al., 1998; Percival et al., 2000). But the most alarming concern is health- associated problems, as there are reports of respiratory (Camper, 1996) and gastroenteritis diseases (Prévost et al., 1998) associated with the potable water main biofilms.

Due to the above reported problems associated with potable water main biofilms, a potable water main system can no longer be considered as the final barrier against contamination of potable water, prior to reaching the consumer's tap (Haas et al., 2002). Therefore, the control of biofilms in new and repaired water mains, installed in water distribution systems, is an important aspect of protecting the quality of water delivered to consumers (US EPA, 1992; Haas et al., 1999; Percival et al, 2000).

There are a number of ways to control the formation or growth of biofilm in new and repaired water mains. The controlling techniques include nutrient control (Camper et al., 2003), use of water main corrosion inhibitors (Geldreich and LeChevallier, 1999) and maintaining adequate disinfectant residual concentration (LeChevallier et al., 1988a; LeChevallier et al., 1991; Ollos et al., 2003; Camper et al., 2003).

Chlorination (chlorine residual in the water distribution system) does not always prevent formation of biofilms on potable water mains (LeChevallier et al., 1996) as it has been shown that biofilm bacteria can attach and grow on any pipe surface even in the presence of chlorine residual in the potable water main (LeChevallier et al., 1996; van der Kooij, 1999 and 2003). Therefore, other biofilm controlling techniques (Percival et al., 2000) such as alternative disinfectants (ozone) need to be investigated (Donlan, 2000).

## 1.2 Problem statement

The construction, rehabilitation and repair of water mains are extremely common activities that occur on a regular basis in all water systems. The installation of new and repaired water mains can be a potential contamination risk to water distribution systems if proper disinfection procedures and standards are not followed (US EPA, 2002). The microbial contamination in new and repaired water mains, installed in water distribution systems, has been associated with several waterborne disease outbreaks in potable water systems. Kirmeyer et al. (2000) reported 35 cases of waterborne disease in the United States of America (USA), from 1920 to 1984, associated with contamination of water main (biofilms). This study (Kirmeyer et al., 2000) also showed the presence of pathogens and fecal coliform bacteria in soil and trench water at water main repair or pipe yard sites. For these reasons, the disinfection of new and repaired water mains prior to installation into water distribution systems is a requirement in the North America (US EPA, 2002), in order to protect the public health (Haas et al., 1999).

The disinfection of new and repaired water mains is conventionally achieved by chlorination, using tablet, continuous-feed, or slug method (American Water Works Association or AWWA, 1999). The choice of a method, as the most suitable method, is decided by the water utility considering various factors such as the length and diameter of the water main, type of water main joints, availability of materials and equipments required for disinfection, skilled personnel, and safety and regulatory concerns regarding the disposal of heavily chlorinated water into the environment.

According to the "AWWA Standards for Disinfecting Water Mains" (AWWA C651-99/ AWWA, 1999), the tablet method consists of attaching 5-g calcium hypochlorite tablets to the walls of dried water mains during the installation and filling the water main with potable water when the installation is complete. The number of tablets depends upon the length (4.0 to 12.2 m) and diameter (100 to 400 mm) of the water main. The water should remain in the water main for at least 24 hours at a minimum water temperature of 5°C and the final solution in the water main should have a chlorine residual of 25 mg/L. The continuous-feed method consists of filling the water main section with chlorinated water

through a temporary connection. The chlorinated water should remain in the water main for at least 24 hours producing a free chlorine residual of not less than 10 mg/L after this holding period. The slug method consists of moving slowly a slug dose of free chlorine (concentration of 100 mg/L) in the water main for a period of not less than 3 h. The free chlorine residual in the water main shall not drop below 50 mg/L at any time during the holding period of 3 hours. After the applicable holding period, the bacteriological testing is used to verify the absence of coliform organisms in the heavily chlorinated water (in the water main). This water is then disposed off into the environment, according to the applicable regulatory requirements. Generally, the chlorine residual of this water is neutralized by using sulfur dioxide, sodium bisulfite, sodium sulfite or sodium thiosulfate.

Denver Water's (the sponsor of this project) current procedure for disinfecting new and repaired water mains involves using the continuous-feed or slug method for large water mains (300 to 2,700 mm diameter) and the tablet method for small water mains (50 to 300 mm diameter). For larger mains, an open trailer-mounted calcium hypochlorite chemical feed system is used (Photo 1-1).

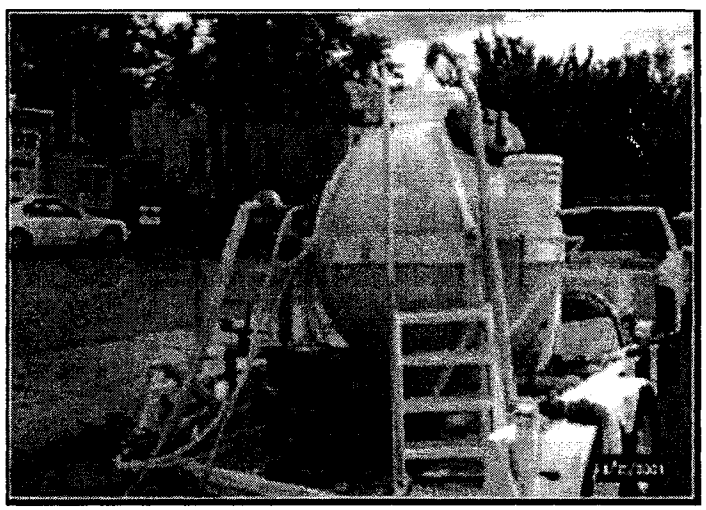

**Photo 1-1:** An open trailer-mounted calcium hypochlorite feed system used by Denver Water for water mains disinfection (Reprinted from Proceedings of 2004 AWWA Water Quality Technology Conference, by permission)

A hypochlorite solution is injected at the upstream end of the water main, a blowoff valve is opened at the downstream end and the solution flows through the water main until a residual of at least 100 mg/L is detected at the downstream blow-off. These valves are then closed and chlorinated water remains in the water main for at least 24 hours. The heavily chlorinated water (in the water main) is then dechlorinated using sodium bisulfite chemical feed system, installed in another trailer, prior to discharge to the environment (Figure 1-1).

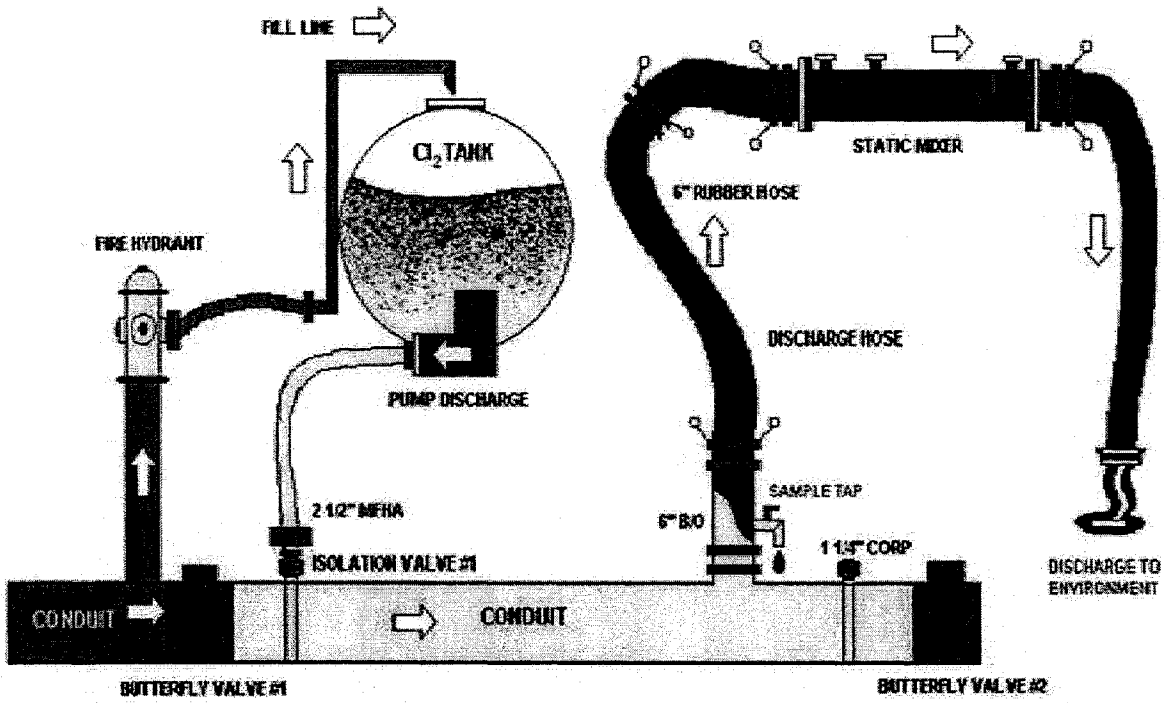


**Figure 1-1:** Denver Water calcium hypochlorite disinfection procedure for water mains (Courtesy of Steve Lohman, Denver Water)

A sample is taken for a coliform test and the main is not placed into service until the test result is determined to be negative (Schulz et al., 2004).

While chlorine-based disinfection methods for water main disinfection are generally effective when properly performed, and conventionally used in the USA, they have several limitations (Schulz et al., 2004):

- the disinfection methods are not linked to a rationale basis for water main disinfection, such as the disinfectant concentration-contact time (Ct) concept used for primary disinfection in drinking water treatment plants; also chlorine dose rates may be overly conservative;

- the heavily chlorinated water from the water main must be dechlorinated prior to discharge to a receiving water body and in practice, 87% of water utilities in the USA do not practice dechlorination;

- the stock solutions of hazardous chlorine chemicals (calcium or sodium hypochlorite) and neutralizing agents are prepared onsite for chlorination and dechlorination steps, which is a tedious and time-consuming procedure;

- the tablet method for disinfection of water mains is not reliable because the chlorine tablets are widely spaced in the water main and, sometimes, these tablets do not fully dissolve in the water main, causing under-dosing of the water main;

- the contact time of minimum 24 hours to ensure adequate disinfection of water mains is problematic for both the water utility and the general contractor when new water mains are installed with tight schedule constraints; and

- there is likelihood of regulating chlorinated discharges by the federal and state regulatory agencies.

Other major concern of using chlorine as a water main biofilm disinfectant is that, a number of microorganisms are found resistant to chlorine disinfection such as oocysts of *Cryptosporidium parvum* and cysts of *Giardia lamblia* and *Legionella pneumophila* (U.S. EPA, 1999). Their possible growth into water main biofilm (Wright, 2000) can further increase their resistance against free chlorine disinfection.

## 1.3 Research Objectives

Due to the above practical limitations and concerns associated with the use of free chlorine as water main (biofilm) disinfectant it was of interest to explore ozone as an alternative disinfectant. Ozone has been used as a primary disinfectant for suspended bacteria in potable water systems, particularly in Europe, since over 100 years (Donlan, 2000). The early application of ozone in the USA was primarily for non-disinfection purposes such as color removal and taste and/or odor control. However, since the implementation of the Surface Water Treatment Rule and the Disinfection By-Product rule, in the USA, ozone usage for disinfection of suspended microorganisms in potable water systems has increased (U.S. EPA, 1999). However, not much progress has been made towards the use of ozone as a biofilm disinfectant in new and repaired water mains.

Needless to say, there is very little information in the literature on the use of ozone as a water main biofilm disinfectant. Therefore, there is great need to explore ozone as a biofilm disinfectant for water mains disinfection. Denver Water, USA is currently evaluating the use of an enclosed trailer-mounted flow-through ozone system for disinfecting their water mains.

The flow-through system is employed because, due to rapid decay of ozone in the water, it is not possible to rely on long holding times, such as the 24-h minimum holding time requirement for disinfection of water mains with chlorine. This system consists of injection of ozonated water at one end of the water main and discharge at the other end. A sufficient ozone dose is applied to maintain an outlet ozone residual of 0.2 to 1 mg/L (Figure 1-2 and Photo 1-2). For small water main lengths, the flow-through disinfection method can be done with one pair of ozone injection and discharge taps. For long water main lengths or large-diameter water mains, two or more pairs of ozone injection and discharge taps may be required to maintain detectable ozone residual along the entire length of the water main. Denver Water has been using both calcium hypochlorite and ozone disinfection systems for water mains disinfection since the year 2003, as a part of their 3-year project, in order to compare the disinfection performance for different sized water mains (Schulz et al., 2004).

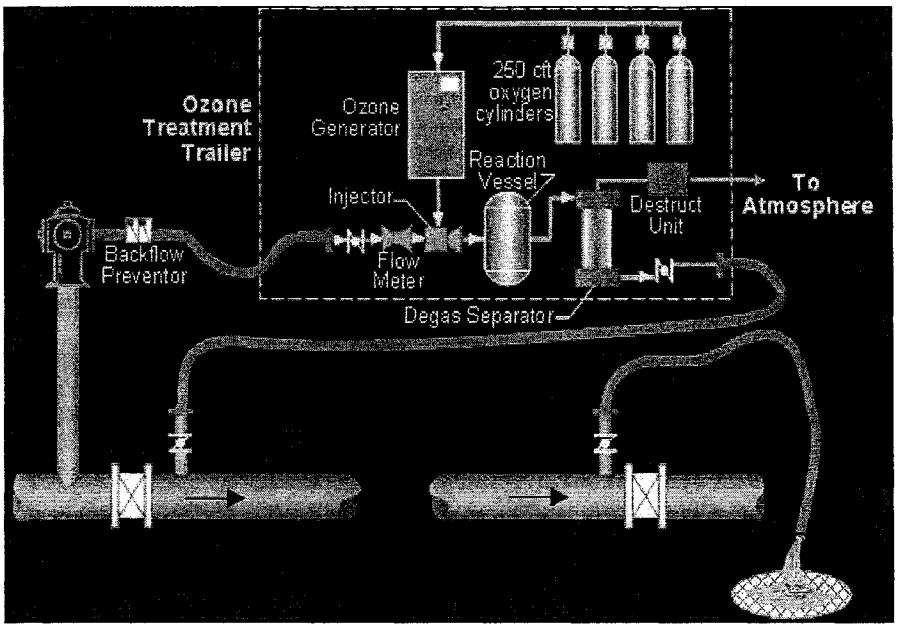


**Figure 1-2:** Denver Water ozone disinfection procedure for water mains (Reprinted from Proceedings of 2004 AWWA Water Quality Technology Conference, by permission)

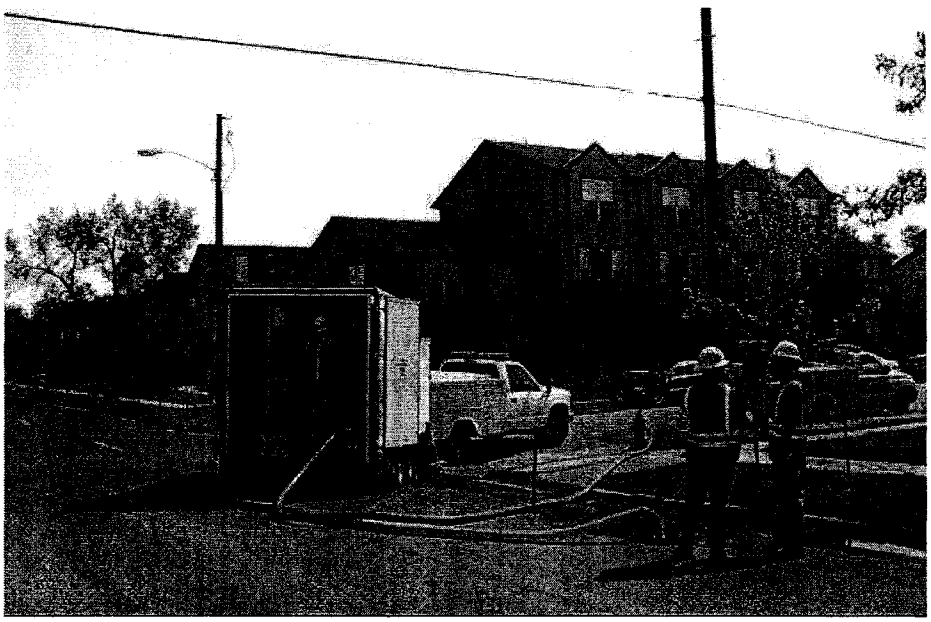

**Photo 1-2:** An enclosed trailer-mounted flow-through ozone disinfection system, used by Denver Water (Courtesy of Steve Lohman, Denver Water)

The potential advantages of using ozone for water mains disinfection, as compared with chlorination, are noted below (Schulz et al., 2004):

- *Effective and rapid disinfection.* Ozone is a powerful disinfectant so water main disinfection can be accomplished in minutes, not hours. Ozone is capable of meeting disinfection targets for suspended bacteria at Ct values around two orders of magnitude lower than for chlorine. Therefore, it is possible to develop a flow-through based ozone disinfection strategy that minimizes disinfection Ct requirement associated with chlorine;

- *Elimination of the dechlorination step.* Ozone decays to oxygen in water, typically in less than 1 hour, depending on temperature, pH, and concentration of ozone-demanding substances in the water. Thus, it is possible to develop a disinfection strategy that allows ozone residual in the water main to decay to oxygen prior to discharging water from the water main to the environment. Alternatively, ozone residuals up to 2 mg/L can be removed rapidly using ascorbic acid tablets, as currently practiced by Denver Water. By comparison, superchlorination of water mains generates chlorine residuals of 25 to 100 mg/L, which cannot be dechlorinated with easy-to-use tablets, but must be dechlorinated using sodium bisulfite at a 2:1 dose-to-residual ratio. This is a more complex procedure, as noted earlier, and few water utilities in the USA practice dechlorination;

- *Environmentally friendly discharges.* Since ozone decays to oxygen and can be easily deozonated, water from the disinfected main can be flushed safely onto streets, sewers, or watercourses without risk of environmental harm. Ozone residuals released into the environment will quickly be consumed upon contact with pavement, dirt, or ultraviolet (UV) light; and

- *No storage or transport of hazardous chemicals.* Ozone cannot be stored, but must be generated at the point of application through an electrical process using

oxygen. The oxygen can either be generated onsite from ambient air using oxygen separation equipment or taken directly from a small gaseous oxygen storage cylinder, as practiced by Denver Water. In both cases, the risk of operator exposure to hazardous chemicals is less than with hypochlorite/bisulfite chemical feed systems.

The objectives of this study therefore included:

1. determination of biofilm and suspended bacteria inactivation using ozone as an alternate disinfectant for water main disinfection under field condition, and comparing the results in terms of log inactivation of Heterotrophic Plate Count (HPC) bacteria corresponding to Ct values;

2. determination of biofilm and suspended HPC bacteria inactivation using free chlorine, under field conditions, and comparing these inactivation results with the ozone inactivation (disinfection) results, respectively; and

3. to determine if biofilm HPC bacteria disinfection is a function of ozone or free chlorine Ct value, to provide a rationale basis for setting water main disinfection requirements in the field.

## 1.4 Scope

This study was sponsored by Denver Water, which supplies potable water in the Denver Metropolitan area. Denver Water was interested to use the ozone in place of free chlorine for the disinfection of new and repaired water mains. The reason was to avoid delays in water mains installation and replacement (24-h holding time for chlorination of water mains) and to avoid dechlorination of chlorinated washed/flushed water prior to disposal. Therefore, this study involved a series of laboratory experiments evaluating the disinfection efficiency of ozone against biofilm HPC bacteria at ambient laboratory temperature (22 ± 1°C), with respect to field conditions (different Ct values for ozone and free chlorine).

The experimental biofilms were grown using an annular reactor (also called as biofilm reactor in this study) with holding capacity of 20 cement-mortar-lined (CML) coupons. The CML coupons were selected to grow biofilms because the water main material used by the Denver Water is coated with the cement mortar.

The biofilm reactor was inoculated with the pipe wash water that was used for washing and cleaning of water mains of Denver Water. This was to ensure that biofilm on coupons was composed of the same mix of naturally occurring microorganisms. The resulting biofilm, however, is probably not identical to the biofilm that growths on the water mains in the pipe yard of the Denver Water.

The ozone disinfection experiments for biofilm HPC bacteria were conducted by immersing a (CML) biofilm coupon in the phosphate buffer and maintaining a target ozone residual concentration of 0.5 to 1.0 mg/L in the phosphate buffer for contact times of 5 to 60 minutes. The free chlorine disinfection experiments for biofilm HPC bacteria were conducted in similar manner but maintaining a target free chlorine residual concentration of 25 mg/L in the phosphate buffer for contact times of 60 minutes to 24 hours. The experimental ozone and free chlorine concentration and contact times (Ct values) were chosen to reflect current field conditions used by the Denver Water. As a consequence, the free chlorine Ct values were 2 orders of magnitude greater than the ozone Ct values.

The disinfection efficiency of ozone and free chlorine for biofilms was determined in terms of log inactivation of HPC bacteria corresponding to Ct values. Ozone and free chlorine disinfection experiments for suspended bacteria were conducted by using diluted pipe wash water, maintaining a target ozone residual concentration of 0.5 to 1.0 mg/L and free chlorine residual concentration of 25 mg/L, respectively. The disinfection efficiency of ozone for biofilms was compared with the disinfection efficiency of ozone for suspended bacteria. Similarly, the disinfection efficiency of free chlorine for biofilms was compared with the disinfection efficiency for suspended bacteria.

Also, the results of ozone disinfection for biofilm and suspended bacteria were compared with the results of free chlorine disinfection for biofilm and suspended bacteria, respectively. The comparison of disinfection results of ozone and free chlorine for biofilm (and suspended bacteria) was necessary to determine how ozone compares to free chlorine as a water main disinfectant under field conditions.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 INTRODUCTION

In the previous chapter it was stated that the main objective of this study was to determine the efficiency of ozone as an alternate disinfectant against biofilm HPC bacteria, under field conditions. It was not the aim of this study to examine the biofilm morphology, biofilm thickness, distribution and composition of microorganisms in biofilm, or other characteristics of biofilms, grown on CML coupons in the laboratory. Therefore, this literature review is mainly focused on the contribution of other researchers in the field of (water main) biofilm disinfection. However, to interpret the disinfection results in terms of biofilm inactivation, it is also important to understand the basics of biofilms, such as biofilm system, biofilm formation or development, and mechanisms of resistance to disinfectants.

### 2.1.1 Biofilm and Biofilm Structure

Since the first study of microbial colonization (biofilms) process on wet surfaces by Zobell (1943), as quoted by Characklis and Marshall (1990), all the researchers agree that the majority of soil and water bacteria can attach to the surfaces of potable water mains resulting in the formation and development of biofilms (LeChevallier, 1987; LeChevallier et al., 1988; LeChevallier et al., 1990; LeChevallier et al., 1991; LeChevallier et al., 1996; Armon, 1997; Stoodley, 1997; Haas, 1999; Keer et al., 1999; Allison et al., 2000; Camper, 2000; Gilbert and Allison, 2000; Lewandowski, 2000; Wimpenny, 2000; Camper et al., 2003; Ollos et al., 2003; van der Kooij, 2003; Hall-Stoodley, 2004; Keinänen, 2004; Menaia and Mesquita, 2004). It is also generally accepted that the formation of biofilms on water main surfaces is mediated through extracellular polymeric substances or EPS (Allison, et al., 2000; Flemming et al., 2000; Gilbert and Allison, 2000; Wright, 2000; Hall-Stoodley et al., 2004). The EPS, also referred to as 'glycocalyx' or 'slime or slime matrix' (Characklis and Marshall, 1990; Geldreich, 1996; Percival et al., 2000; Allison et al., 2000; Hall-Stoodley et al., 2004), can be considered as "building blocks" of biofilms, providing structural and functional integrity to biofilms (Flemming et al., 2000).

The EPS, ~ 50 to 90% of the biofilm mass (Flemming et al., 2000, Percival et al., 2000), are mainly responsible for the internal structure and physico-chemical properties of biofilms (Percival et al., 2000). The EPS are not only responsible for formation and development of biofilms, by adhering other microorganisms, but also provide a medium of interaction among microbial cells (Flemming et at., 2000; Allison et al., 2000; Percival et al., 2000). Such interaction among biofilm microbial cells may result in generating a very stable and strong functional capability in biofilms, different from suspended microorganisms, that can provide resistance to water disinfectants (Allison et al., 2000).

It was believed that biofilms are often "patchy" in appearance (Characklis and Marshall, 1990; Percival et al., 2000) and "structurally heterogeneous" (Lewandowski, 2000) but sometimes provide a uniformly distributed layer of microbial species (Lewandowski, 2000; Percival et al., 2000). The new research has confirmed that biofilms are structurally heterogeneous and complex in nature (Hall-Stoodley et al., 2004). The algal biofilms (termed as "mats") can be up to 400 mm thick (Characklis and Marshall, 1990). However, in water mains, isolated from the potable water systems for repair and new water mains left over in a pipe yard for some time prior to installation, biofilm thickness is only a few hundred micrometers (Wimpenny, 2000).

### 2.1.3 Microorganisms in a Biofilm (Biofilm-Forming Bacteria)

Analyses of scraped biofilm samples from water main walls reveal the presence of various microorganisms in a biofilm. However, bacteria normally constitute the predominant microorganisms in most of potable water main biofilms. Heterotrophic bacteria are present in the largest numbers in biofilms (Characklis et al., 1990; Geldreich, 1996). Biofilms in water mains have the potential to harbor pathogenic microorganisms and opportunistic pathogens such as *Pseudomonas aeruginosa, Legionella pneumophila, Flavobacterium* spp., *Klebsiella* spp., *Helicobacter pylori, Escherichia coli* O157:H7, *Vibrio cholerae,* and *Salmonella typhimurium* (Geldreich and Rice, 1987; LeChevallier et al., 1988a; Characklis et al., 1990; LeChevallier et al., 1990; Geldreich, 1996; Armon et al., 1997; Mackay et al., 1998; Camper, 2000; Storey et al., 2004), if proper water main disinfection procedures and standards are not followed (AWWA, 1999).

### 2.1.4 Biofilm System

A biofilm system can have as many as five compartments (Figure 2-1):

**i.** the substratum

**ii.** the base (subsurface) film

**iii.** the surface film

**iv.** the bulk liquid

**v.** the gas

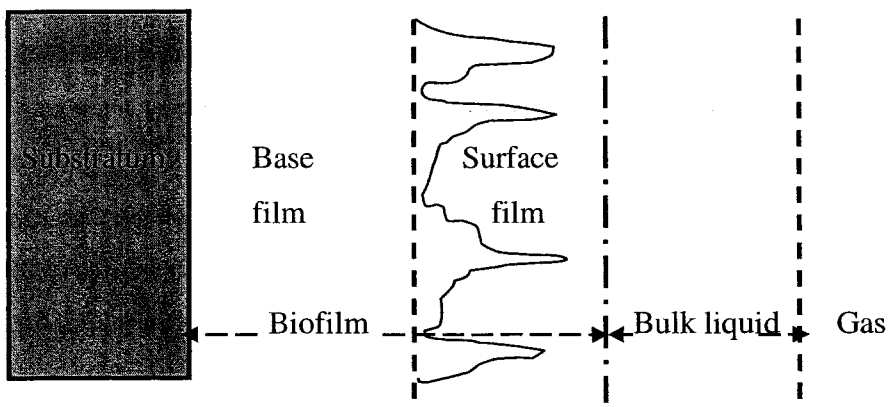


**Figure 2-1:** The biofilm system (Redrawn from Characklis and Marshall, 1990)

The substratum, the supporting surface, plays a key role in the formation of biofilm and its subsequent growth rate. The base film generally dictates the biofilm growth and its thickness. The surface film acts as an intermediate compartment between the bulk liquid and the colonized base film. Both the base film and the surface film compartments are together known as the 'biofilm compartment' as these compartments constitute the 'biofilm' (Characklis and Marshall, 1990).

### 2.1.5 Biofilm Formation/Development

The researchers are generally agreed that the biofilm development is the net result of five stages (Characklis, 1990; Geldreich, 1996; Percival et al., 2000; Wimpenny, 2000; Hall-Stoodley et al., 2004):

i. development of a "conditioning film"

ii. reversible adsorption or loose attachment of cells via transport mechanisms

iii. irreversible adsorption or adhesion of cells via EPS

ii. formation of microcolonies and subsequent development of mature biofilm

v. detachment of some biofilm cells

Conditioning of water main surfaces, even clean, occurs after adsorption of organic molecules on substratum (van Hoogmoed et al., 2000). The "conditioning film", 30 to 80 nm thick, plays a major role in the attachment of "pioneer" cells (loosly attached) on the substratum followed by "adhesion" of cells (Percival et al., 2000; Hall-Stoodley et al., 2004). These pioneer cells then reproduce, grow, and multiply forming microcolonies and subsequent development of mature biofilms (Percival et al., 2000; van Hoogmoed et al., 2000; Hall-Stoodley et al., 2004). Bacterial detachment or sloughing can occur in mature but unstable biofilm cells (Wimpenny, 2000; van Hoogmoed et al., 2000).

### 2.1.6 Factors Favoring Biofilm Growth

The following factors may be responsible for biofilm formation and may enhance biofilm growth in water distribution systems:

#### *i. Environmental Factors*

Water temperature is considered as the most important environmental factor responsible for biofilm growth in water mains (LeChevallier, 1988a; LeChevallier et al., 1991; Camper, 2000; Ollos et al., 2003; Menaia and Mesquita, 2004). It is reported that biofilm growth is rapid at higher temperatures (>15°C) (Fransolet et al., 1985; Donlan and Pipes, 1988).

#### *ii. Nutrient Availability*

The presence of biofilm growth-promoting nutrients such as carbon (assimilable organic carbon, AOC), nitrogen (ammonia nitrogen), and phosphorus (orthophosphate) in the treated water in a ratio of about 100:10:1 may promote bacterial growth in potable water mains systems (LeChevallier, et al., 1991; van der Kooij, 1992 and 1999; Prévost et al.,

1998; Camper, 2000; Gagnon et al., 2000; Menaia and Mesquita, 2004). Rainfall events, causing increase in runoff-associated nutrients, may also promote biofilm growth (Camper et al., 1999; Ollos et al., 2003).

### *iii. Water Main Construction Materials and Appurtenances*

Researchers have shown that all water main construction materials including cement, cast iron, copper, stainless steel, polyvinyl chloride (PVC), polyethylene (LeChevallier et al., 1991; Geldreich, 1996; Keer et al., 1999; Camper, 2000; Ollos et al., 2003; Menaia and Mesquita, 2004), and plumbing materials such as rubber, silicon, and bituminous coatings can contribute to bacterial (re)growth in the form of biofilms (Rogers, 1994).

### *iv. Disinfectant Type and Residual Concentration*

There are a number of reports that indicate the occurrence of biofilm growth in the water mains even in the presence of a chlorine or monochloramine residual (LeChevallier et al., 1991; Prévost et al., 1998; Percival et al., 2000; Ollos et al., 2003; van der Kooij, 2003).

### *v. System Hydraulics*

It is generally believed that potable water main system hydraulics can enhance the formation of biofilms (LeChevallier et al., 1988a; Menaia and Mesquita, 2004). High flow rate in the system can result in the increased nutrient transport to pipe surface and subsequently increased biofilm formation, and biofilm detachment or sloughing (Donlan and Pipes, 1988; Camper et al., 1999).

## 2.1.7 Controlling Biofilm Formation

Biofilm formation in a water main can be controlled by a number of ways. The following are the controlling methods as suggested by various researchers:

**i.** *Controlling nutrients* such as minimizing the amount of AOC entering the new and repaired water mains, installed in a water distribution system (LeChevallier et al., 1991; Prévost et al., 1998; Camper, 2000; Menaia and Mesquita, 2004),

**ii.** *Corrosion (phosphate-based) inhibitors* as a water main coating material, providing increased disinfectant effectiveness (LeChevallier et al., 1990; Geldreich and LeChevallier 1999; Volk et al., 2000; Keinänen et al., 2004),

**iii.** *Maintaining adequate disinfectant residuals* throughout new and repaired water main system (Prévost et al., 1998; Ollos et al., 2003; Camper et al., 2003; van der Kooij, 2003)

**iv.** *Controlling system hydraulics* (regular flushing of water mains and periodic pigging of localized areas susceptible to biofilm growth) can minimize the existence of biofilm formation (US EPA, 1992; van der Kooij et al., 1999), and

**v.** *Reducing microbial entry* to the water main with cross-connection control, replacement of damaged water mains, and disinfection of new pipe materials and pipe fittings prior to installation into potable water system (US EPA, 1992; AWWA, 1999; Geldreich and LeChevallier, 1999; van der Kooij et al., 1999).

### 2.1.7 Mechanisms of Resistance to Disinfectants

A number of researchers have investigated the resistance mechanisms of biofilms against disinfectants and agreed that biofilms provide a resistance to water disinfectants (LeChevallier et al., 1987; LeChevallier et al., 1991; deBeer et al., 1994; Stoodley et al., 1994; Huang et al., 1995; Stoodley et al., 1994; Chen and Stewart, 1996; Heinzel, 1998; Allison et al., 2000; Camper, 2000; Gilbert and Allison, 2000; Hall-Stoodley et al., 2004).

Various hypotheses, concerning biofilm resistance against disinfectants, were therefore reported by these researchers. However, according to the recent developments the area of biofilm resistance to disinfectants, three mechanisms can be considered as a possible explanation for the resistance of biofilms to disinfectants (Hall-Stoodley et al., 2004):

**i.** *the reaction-diffusion interaction with the EPS* causing neutralization or reduction of disinfectant (concentration) at the biofilm-liquid interface or into biofilms, subsequently resulting in no or less inactivation of cells within the biofilm,

**ii.** *the "physiological state" of biofilm cells* generating metabolically dormant cells in biofilms, protecting them from disinfectant (concentration) even on penetration of disinfectant into biofilm matrix, and

**iii.** *the growth of "resistant phenotypes or persisters" in the biofilm* resulting in biofilm resistance against disinfectants.

### 2.1.9 Growing Biofilms

There are various techniques of growing biofilms as reported by a number of researchers. Gary et al. (2001) grew three-species biofilm (*P. aeruginosa*, *P. fluorescens*, and *Klebsiella pneumoniae*) on glass slides in laboratory-scale re-circulating biofilm reactors at room temperature (25°C). Typical cell concentrations of their 4-day biofilms were between $1 \times 10^7$ and $1.4 \times 10^8$ colony forming units (CFU) per $cm^2$ of substrate surface. Wingender and Flemming (2004) grew biofilms on stainless steel, copper, PVC, and polyethylene coupons exposed to unchlorinated ground water for 12 to 18 months. They reported the biofilm HPC bacteria concentration on the substrate to be in the range of 9 x $10^3$ and 7 x $10^5$ CFU/$cm^2$. The difference in the concentrations of microorganisms on the substrates reported between these two studies reflects differences in the growth conditions. In the Gary et al. (2001) study, the substrates were exposed to a nutrient rich medium inoculated with laboratory preparations of bacteria, while in the Wingender and Flemming (2004) study, the substrates were exposed to naturally-occurring bacteria in unchlorinated ground water.

## 2.2 BIOFILM DISINFECTION

### 2.2.1 Introduction

It is earlier stated that the major concern related to biofilms in potable water mains is potential health hazards due to presence of pathogenic microbes in biofilms (Camper,

1996 and 2000; Prévost et al., 1998; Kirmeyer et al., 2000). Therefore, the disinfection of biofilms in new and repaired water mains becomes important to avoid any subsequent major health problem (US EPA, 1992; Haas et al., 1999).

### 2.2.2 Biofilm and Suspended Bacteria Disinfection by Chlorine

#### *a. Why Chlorine*

Chlorine is used as an effective disinfectant for bacteria. Also, it can be easily applied, measured, and controlled and, above all, is relatively cheap and readily available.

#### *b. Chlorine Chemistry*

When chlorine is added to water, hypochlorous acid (HOCl) forms:

$Cl_2 + H_2O \leftrightarrow HOCl + H^+ + Cl^-$ $\quad pKa_1 = 3.33$ ($K \approx 4.5 \times 10^{-4}$ at 25°C)

Depending on the pH value, HOCl partly dissociates to hypochlorite ($OCl^-$) ion:

$HOCl \leftrightarrow H^+ + OCl^-$ $\quad pKa_1 = 7.53$ ($K \approx 2.9 \times 10^{-8}$ at 25°C)

HOCl (electrically neutral) and $OCl^-$ ions (electrically negative) combine to form free available chlorine. Both substances have very distinctive behavior. HOCl is more reactive and is a stronger disinfectant than $OCl^-$ ($OCl^-$ is repelled by negatively charged bacterial cell wall). At 20°C and pH 6, 7, and 8, HOCl constitutes 97.5, 79.3, and 27.7%, respectively of free chlorine. Therefore, at high pH, the effectiveness of free chlorine is reduced (U.S. EPA, 1999; White, 1999).

#### *c. Mode of Action (Inactivation Mechanisms)*

A number of researchers have proposed mechanisms for the inactivation of suspended bacteria by chlorine. McFeters and Camper (1983) found that the chlorine on reaction with cell surface enzymes reduced metabolic activity of bacteria. They showed that chlorine-injured cells lost their ability to transport glucose and amino acids to the cell membrane. Sletten (1974) reported that HOC1 is more effective than $OC1^-$ in inactivating suspended bacteria. Because, HOCl is electrically neutral and therefore can penetrate easily through negatively charged bacterial cell wall, rendering them inactive.

Armon et al. (1997) showed that chlorine, in the form of hypochlorite, can inactivate biofilm cells by depolymerizing the EPS in the biofilm as well as can cause detachment of biofilm cells.

### *d. Disinfection Efficiency*

Camper (1996) has reported that by maintaining free chlorine residual of about 0.2 mg/L, biofilm concentration on pipe surfaces was reduced. Armon et al. (1997) conducted a study on laboratory biofilms, grown on glass, galvanized iron, and PVC coupons. Exposing the biofilms to 1 to 3 mg/L of chlorine, they found that chlorine was relatively more effective against the biofilms grown on glass and galvanized iron coupons in comparison to PVC coupons.

A similar study, on efficiency of chlorine against biofilms developed on various materials (copper, galvanized iron, and PVC), was conducted by LeChevallier et al. (1996). They also compared the disinfection efficiency of free chlorine (HOCl) with monochloramine ($NH_2Cl$). They found that the pipe material has a considerable effect on the efficiency of chlorine against biofilms. Biofilm bacteria grown on copper or PVC pipe surfaces were easily inactivated by a 1 mg/L residual free chlorine and monochloramine. However, maintaining 3 mg/L free chlorine residual in galvanized iron pipe biofilms for two weeks did not produce any significant log inactivation. In comparison, treating galvanized iron pipe biofilms with same concentration and contact time more than 3 log inactivation was achieved. They reported that the relative ineffectiveness of free chlorine against biofilms was due to chlorine demand of iron substratum.

Momba et al. (1998 and 2003) evaluated several disinfectants (chlorine, chloramine, UV irradiation, and hydrogen peroxide) for biofilm growth in potable water mains, by growing biofilm HPC bacteria on stainless steel and cement coupons in the laboratory. They reported more than 2 log inactivation of biofilm HPC bacteria by all disinfectants, within 5 minutes contact time. However, a longer contact time was required for disinfection with chloramine (24 hours) and hydrogen peroxide (72 hours). To compare

the effect of chlorine and chloramine on biofilm HPC bacteria, Neden et al. (1992) divided a section of Greater Vancouver Water District distribution system in three areas, viz. a control area (no secondary disinfectant) and one area disinfected with free chlorine and other with chloramines. They found chloramine was relatively effective in controlling biofilm HPC bacteria.

Comparing the disinfection efficiency of chlorine and chloramines against biofilm and suspended bacteria, LeChevallier et al. (1988a and 1988b) reported that biofilm bacteria are up to relatively 150 times and 100 times more resistant to free chlorine and monochloramine, respectively.

### 2.2.3 Biofilm and Suspended Bacteria Disinfection by Ozone

#### *a. Why Ozone*

Ozone is a powerful oxidant that has been documented as an effective disinfectant against number of pathogenic organisms including bacteria, protozoa, and viruses, in suspension (U.S. EPA, 1999).

#### *b. Primary Uses of Ozone*

Ozone is used in drinking water treatment for a variety of purposes viz. **(i)** disinfection (U.S. EPA, 1999) including inactivation of *Escherichia coli* (Finch et al., 1988; Hunt and Mariñas, 1997 and 1999) and inactivation of *Cryptosporidium parvum* oocysts (Rennecker et al., 2000; Driedger et al., 2001; Corona-Vasquez et al., 2002); **(ii)** coagulation and filtration improvement (Reckhow et al., 1993); **(iii)** inorganic pollutant oxidation, including iron, manganese, and sulfide (Geldreich, 1996; Camel and Bermond, 1998); **(iv)** organic micropollutant oxidation, including taste and odor compounds (Geldreich, 1996); and **(v)** color removal and disinfection byproduct precursor control (Georgeson and Karimi, 1988).

#### *c. Ozone Chemistry*

For application in water treatment, ozone is usually generated by the corona discharge technique. In this technique, dry (pure) oxygen gas is passed between two electrically

charged, narrowly spaced electrodes, converting part of the oxygen to ozone (Carlins and Clark, 1982; Rice, 1986; Percival et al., 2000):

$$O + O_2 \quad \leftrightarrow \quad 2O_3$$

Basic chemistry research has shown that, in aqueous solution, ozone can react by either direct oxidation of compounds by molecular ozone and/or oxidation of compounds by hydroxyl free radicals produced during the decomposition of ozone (U.S. EPA, 1999).

### *d. Mode of Action (Inactivation Mechanisms)*

Inactivation of suspended bacteria by ozone is attributed to an oxidation reaction. The first site of attack appears to be the bacterial membrane either through the glycoproteins, glycolipids, or through certain amino acids such as tryptophan. In addition, ozone disrupts enzymatic activity of (suspended) bacteria by acting on the sulfhydryl groups of certain enzymes. Beyond the cell membrane and cell wall, ozone may act on the nuclear material within the cell (U.S. EPA, 1999). As there is very little information available on the use of ozone as a water main biofilm disinfectant therefore, the inactivation mechanism of ozone against biofilms is still not clear.

### *e. Disinfection Efficiency*

The disinfection effectiveness of ozone varies considerably with the type of microorganisms. It is reported that viruses and encysted protozoa are more resistant to ozone than vegetative bacteria (Craik, 2001).

Ozone is found very effective against suspended bacteria. For example, *Legionella pneumophila* levels were reduced by greater than 2 logs with a contact time of 5 minutes at ozone concentration of 0.21 mg/L (U.S. EPA, 1999). Gyüre'k and Finch (1998) used ozone to inactivate suspended HPC bacteria in 0.05 *M* (pH 6.9) phosphate buffer at 22°C in bench-scale, 250 mL batch reactors. Applying ozone doses of 0.31 to 2.13 mg/L for contact times of 0.58 to 14.93 minutes with final ozone residuals between 0.08 to 1.85 mg/L, they observed 2 to 4 log inactivation of suspended HPC bacteria.

Momba et al. (1998) evaluated the effectiveness of ozone against biofilm bacteria, by growing biofilms on stainless steel and cement coupons. Applying the ozone Ct value of 13 mg × min/L (2.6 mg/L for 5 minutes), they found 4 log inactivation of biofilm HPC bacteria.

## 2.3 HPC BACTERIA AS TEST ORGANISMS

There are a variety of organisms to be found in water supply system. Many of these organisms pass through various treatment processes and others, such as HPC bacteria, enter the water system in open finished water reservoirs, during line repairs, in backflows from pipelining projects and new pipe networks installation. Most often these organisms are not of immediate public health concern, but upon multiplication in a water main habitat, as biofilm, can become as an opportunistic pathogen threat (Geldreich, 1996).

The term ''heterotrophic bacteria'' includes all bacteria that use organic nutrients for growth and multiplication. Many of these are "aerobic", and are universally present in all types of water, food, soil, vegetation, and air. HPC bacteria are defined in this study as those microbes enumerated on R2A medium incubated at 20 to 37°C for 3 to 7 days, depending upon research objectives (Bartram et al., 2003). It is widely accepted that the analyses for HPC bacteria in water distribution systems, installed with new and repaired water mains, can be helpful in assessing changes in finished water quality during water main system repair and rehabilitation (Geldreich, 1996; Carter et al., 2000; Robertson et al., 2003; van der Kooij, 2003).

Geldreich (1990) has shown that R2A medium yields significantly higher bacterial counts than Plate Count Agar. The membrane filter (MF) method is accepted as the most flexible method for the HPC determination. The MF method permits the analysis of sample volumes from < 1.0 ml to as much as 10.0 L, depending on the water quality. Thus, very low concentrations of bacteria in a water sample can be detected (Reasoner, 1990).

It should be mentioned here that various researchers have reported the use of HPC bacteria as a tool to examine and characterize distribution systems (biofilms). These researchers include Olson (1982), LeChevallier et al. (1988a), LeChevallier et al. (1990) and McFeters (1990). LeChevallier et al. (1990) tested over 80 HPC bacteria representing all colony morphologies observed in drinking water samples.

McFeters (1990) has reported the use of 3 to 7 days as incubation time and 20 to 37°C as incubation temperature for enumeration of HPC bacteria, using membrane filter method. It was concluded that, in general, the longer the incubation, the higher the viable HPC bacteria count.

In this study, the HPC bacteria were enumerated at 25°C for 5 days because these incubation conditions reflect the field conditions at Denver Water.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 MATERIALS

For biofilm disinfection experiments, the biofilm samples were grown on CML coupons in a biofilm annular reactor (model: 1120 LS Laboratory Annular Reactor, Biosurface Technologies Corporation, Bozeman, Montana, USA). The coupons (in the reactor) were exposed to pipe wash water that was used for washing and cleaning of water mains of the Denver Water.

To monitor the quality of pipe wash water, for biofilm growth, and to carry out biofilm and suspended bacteria disinfection experiments by ozone and free chlorine the following materials and chemicals were prepared:

- Deionized (DI) laboratory water
- Ozone-demand free (ODF) water
- Oxidant-demand free glassware
- Acid-washed glassware
- Chemical oxygen demand (COD) reagents including standard potassium dichromate digestion solution (0.01667 *M*), sulfuric acid reagent, and potassium dihydrogen phthalate (KHP) standard
- Total organic carbon (TOC) reagents including organic and inorganic carbon stock solutions, and potassium persulfate ($K_2S_2O_8$ 2% w/v) solution
- Phosphate buffer (0.05 *M*) of pH 6.0, 6.9 and 9.0
- Sodium formate (1.0 *M*) solution
- Sodium thiosulfate (0.1 *N* $Na_2S_2O_3.5H_2O$) solution
- Ozone measurement reagents including indigo stock and working solutions, and malonic acid reagent
- Free chlorine measurement reagents including potassium permanganate ($KMnO_4$) stock and working solutions, and standards

Refer to Appendix "A" for preparation details of above materials and chemicals.

## 3.2 ANALYTICAL METHODS

All water quality parameters, except HPC bacteria, were analyzed according to "Standard Methods for the Examination of Water and Wastewater, 20th edition" (Eaton et al., 1999). Table 3-1 summarizes the standard methods used:

**Table 3-1:** Summary of standard methods used

| Parameter | Standard Method No. | Abbreviation | Unit |
|---|---|---|---|
| pH value | 4500-$H^+$ | pH | N/A |
| Temperature | 2550 | N/A | °C |
| Chemical oxygen demand | 5220 D | COD | mg $O_2$/L |
| Total Organic Carbon | 5310 B | TOC | mg/L |
| Ozone residual | 4500-$O_3$ B | $O_3$ residual | mg $O_3$/L |
| Chlorine residual | 4500-$Cl^-$ G | $Cl_2$ residual | mg $Cl_2$/L |

N/A not applicable

### 3.2.1 Modified HPC Bacteria Enumeration Method

HPC bacteria were enumerated using a "modified" HPC (25°C, 5-day incubation) method (Haas et al., 1999). The "standard" method for HPC requires 48 hours incubation at 37°C. However, researchers have enumerated HPC bacteria by incubating them at 20 to 37°C for 3 to 7 days (McFeters, 1990; Bartram et al., 2003).

The "modified" method was used in this study because water temperature in water mains of Denver Water is about 25°C (Refer Appendix "A" for further details). The 5-day incubation was selected to reflect maximum time period for which water mains remain in Denver Water pipe yard before installation into distribution system.

## 3.3 BIOFILM REACTOR SETUP AND OPERATION

The details of biofilm annular reactor setup and operation are provided in Appendix "B". However, a brief description is stated as follows:

A 900 mL liquid capacity presterile biofilm annular reactor was used to grow the biofilm on presterile CML coupons (15 cm long x 0.5 cm wide with 0.5 mm thick cement mortar layer), provided by Biosurface Technologies Corporation (Bozeman, Montana, USA). The reactor consisted of a stationary outer cylinder and a rotating inner drum that could accommodate up to twenty rectangular test coupons mounted vertically (Photo 3-1). Refer Appendix "C" for all the physical dimensions of the reactor, as measured.

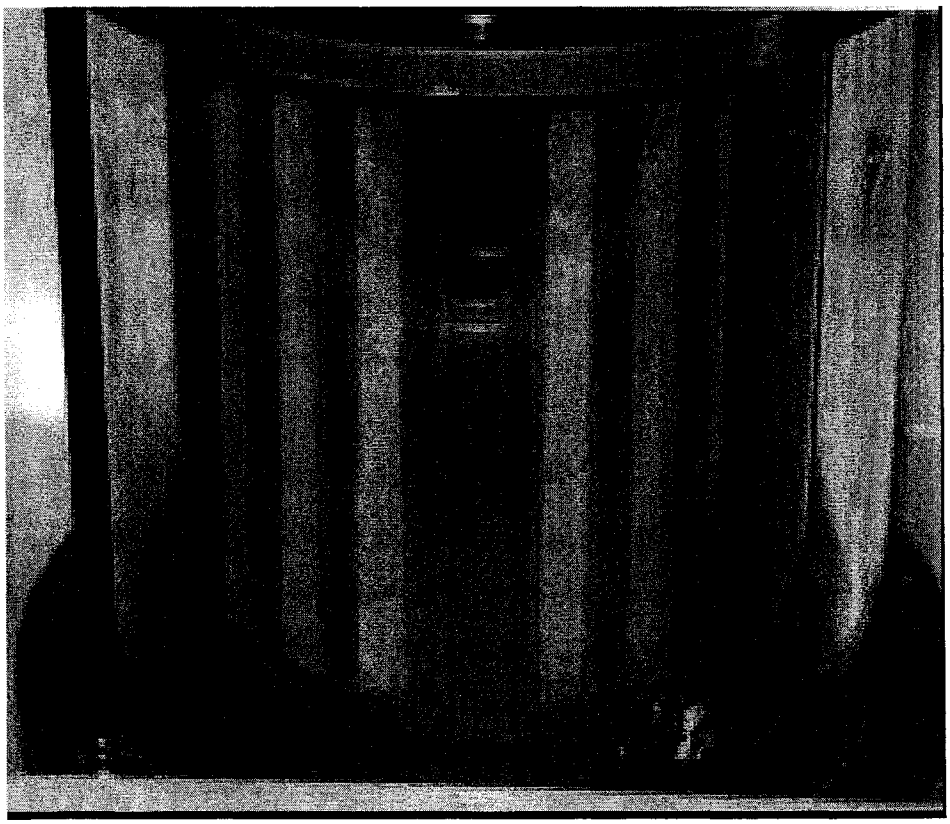

**Photo 3-1:** Biofilm annular reactor containing CML coupons with biofilm growth

During the reactor operation, about 10 to 20 L of the pipe wash water, contained in an external reservoir, was re-circulated continuously through the reactor (rotating at 10 rpm) in a closed loop at a rate of 4.5 mL/min. The pipe wash water was sent by Denver Water in a 10 L plastic container, as needed, through courier. The reservoir was aerated to provide mixing and to ensure sufficient aqueous oxygen for aerobic biofilm growth (Figure 3-1 and Photo 3-2).

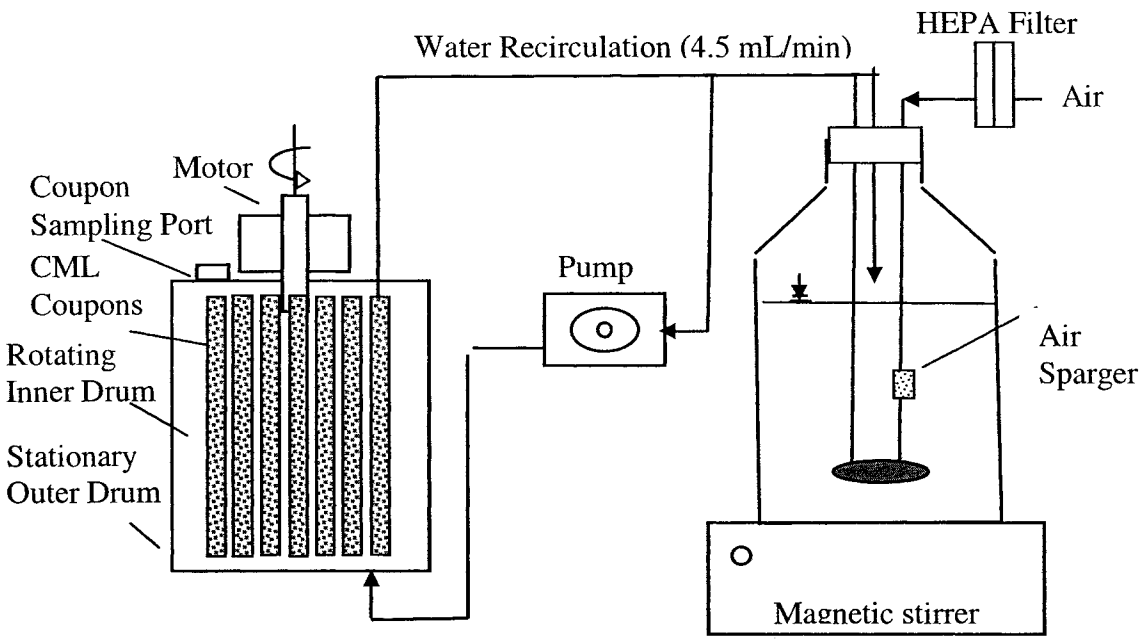


**Figure 3-1:** Schematic of operational setup of biofilm annular reactor

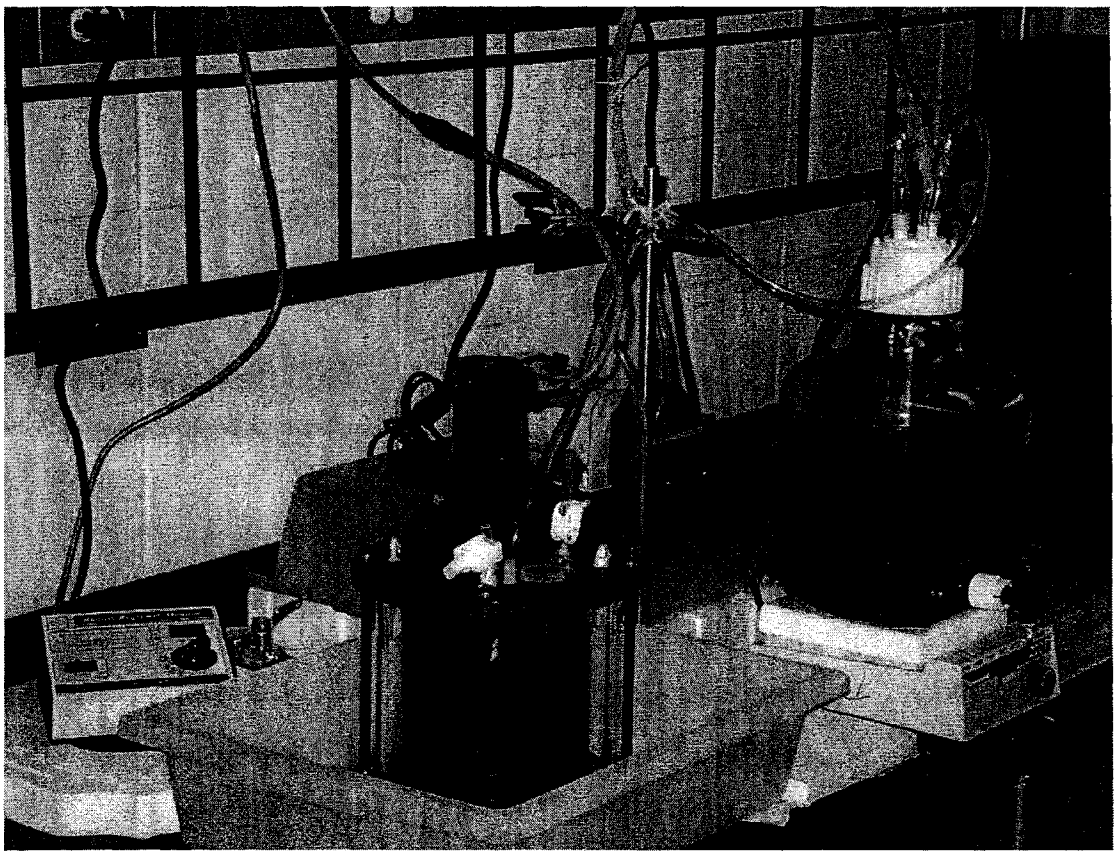

**Photo 3-2:** A view of operational setup of biofilm annular reactor

The pipe wash water was dechlorinated using 0.1 *N* sodium thiosulfate solution prior to exposing to the annular reactor (biofilm coupons). The pipe wash water in the external reservoir was partially replaced roughly every three weeks through a draw and fill procedure. The wash water was not completely replaced to avoid any shock to biofilm HPC bacteria subject to change in wash water quality, received from Denver Water. The pipe wash water in the external reservoir (recirculated water) was monitored daily for COD (March 3 to July 19) and TOC (March 3 to May 7) in order to make sure the availability of organic substrate in the biofilm reactor for biofilm growth. The samples were acidified and stored at a temperature of 4°C for maximum of one week, if could not be analyzed on the same day. The water could not be monitored for TOC beyond May 7 due to malfunctioning of TOC equipment (Model: Dohrmann DC-80 Carbon Analyzer, Xertex Corporation, Santa Clara, California, USA).

## 3.4 DISINFECTION EXPERIMENTS

### 3.4.1 Collection of biofilm samples

#### *i. Biofilm Coupon Removal*

The (CML) biofilm coupons were periodically removed from the annular reactor with the help of a sterilized hooked tool, after turning off the motor drive and the flow pump. The top sample port was removed slowly to avoid flow-burst due to built-up pressure. The retrieved coupon was replaced with a new sterile coupon.

One-half of the coupons was used for determination of initial biofilm concentration ($CFU/cm^2$) and the other half was used in disinfection experiments. In later stages of this study, some of the coupons were cut in half length-wise before inserting into the annular reactor, mainly to increase the number of biofilm samples available for disinfection experiments.

The biofilm disinfection experiments were carried out in randomized order in terms of exposure of upper or lower coupon section to disinfectant, to reduce the effect of nuisance variables (like non-uniform growth) on the interpretation of the disinfection results.

*ii. Determination of Initial Biofilm Density*

One-half of the biofilm coupon was scraped with a sterile rubber policeman to remove the attached bacteria (biofilm). The scraped material was collected into 50 or 100 mL of sterilized phosphate buffer in a beaker. The contents of the beaker were homogenized for 3 minutes using a laboratory tissue homogenizer (PowerGen 700D, Fisher Scientific Ltd., Hampton, New Hampshire, USA) at 25,000 revolutions per minute. The purpose of the homogenization step was to break up bacterial aggregates and to disperse the bacteria in the phosphate buffer. This procedure (of homogenization) was standardized for all biofilm disinfection experiments.

The HPC concentration in the phosphate buffer was determined using the modified HPC method. The coupon with the remaining biofilm was then used in disinfection experiments.

*iii. Biofilm age*

Biofilms of various ages (period of growth in the annular reactor) were used in this study. For ozone experiments, the biofilm age ranged from 3 to 17 weeks. For free chlorine disinfection experiments, the biofilm age ranged from 6 to 21 weeks. For the purpose of this study, biofilms that were between 16 and 21 weeks of age were referred to as 'mature biofilms'. Biofilms that were 3 to 7 weeks old were referred to as 'immature biofilms'. These are arbitrary designations.

### 3.4.2 Disinfection of Biofilms

*i. Ozone Experiments*

For disinfection experiments with ozone, the biofilm sample was inserted into between 200 and 460 mL of ODF phosphate buffer in 500 mL or 1,000 mL ODF reactors (borosilicate Erlenmeyer flask containing a Teflon-coated magnetic stir bar, previously made ODF and sterilized) in such a way that the biofilm on the coupon was completely submerged. The size of the reactor flasks was mainly selected based upon the final volume of solution in the reactor for ozone contact time.

The liquid contents of the reactor were stirred by a magnetic stirrer (Thermix 120 M, Fisher Scientific Ltd., Hampton, New Hampshire, USA), using a Teflon-coated magnetic stir bar, previously made ODF and sterilized. The stirring was aimed to ensure biofilm surface is exposed to bulk ozone solution but to minimize loss of ozone due to vaporization.

The biofilm disinfection experiments were run in randomized order, using the MS®Excel Randomization function, in terms of contact time and biofilm coupon section (upper or lower half). For ozone disinfection experiments, an ozone stock solution (concentration of approximately 25 mg/L) was metered into the reactor flask continuously using a peristaltic pump (#7553-80, Cole-Parmer Instrument Company, Vernon Hills, Illinois, USA) to provide the desired ozone residual concentration (0.5 to 1.0 mg/L) as required by Denver Water for their field application.

The concentrated ozone stock solution was prepared by bubbling the ozonized gas through cold DI water (450 mL in 500 mL gas absorption bottle) for about 20 minutes. The ozone gas (3 to 5% v/v) was generated from extra-dry oxygen using a water-cooled, corona discharge generator (Welsbach T-816, Welsbach Ozone Systems Corporation, Sunnyvale, California, USA). In a few trial experiments, an aliquot of concentrated ozone stock solution was also added to the reactor at the start of the experiment to satisfy the immediate ozone demand.

The ozone stock solution concentration was measured by the direct UV method, at an absorbance of 260 nm ($A_{260}$), using an Ultraspec 2000 spectrophotometer at 1 cm path length and molar absorptivity of 3,300 $M^{-1}$ $cm^{-1}$ (Pharmacia Biotech Ltd., Cambridge, UK).

The ozone concentration in the reactor flask was monitored in real-time by re-circulating a portion of flask solution through a 35 $\mu$L, 10 mm path length flow cell, measuring $A_{260}$ on Hewlett-Packard (model 8452A) diode-array spectrophotometer (Figure 3-2).

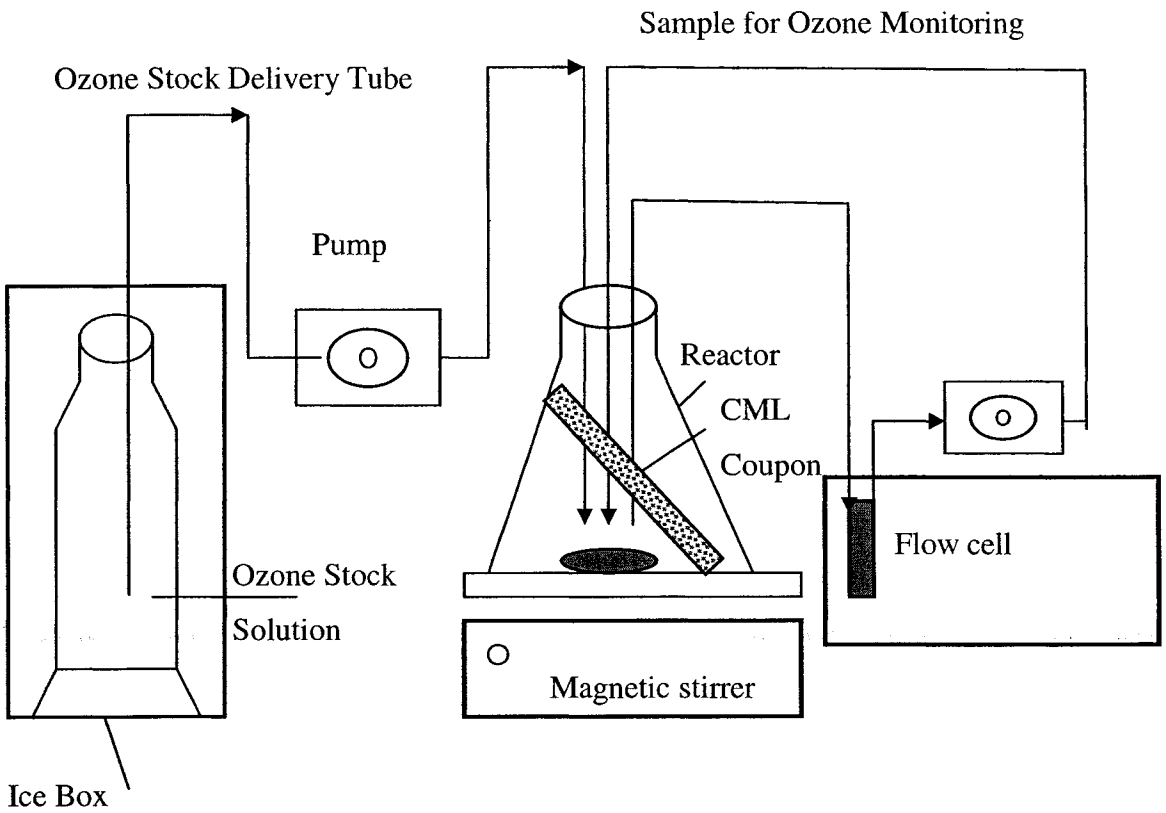


**Figure 3-2:** Schematic of the apparatus used for exposure of coupons to ozone

The $A_{260}$ measurements (a convenient way to monitor real-time ozone residual in the reactor solution) were supplemented by periodically extracting samples for ozone measurement using the indigo colorimetric method ($A_{600}$) with a 1 cm path length cuvette on Ultra spec 2000 spectrophotometer.

The major reason to measure concentration in the solution in the reactor using the indigo colorimetric method was that this method is used as a standard method for measuring ozone concentration in water. Also, this method is relatively free of interferences in comparison to direct UV method. The UV method ($A_{260}$) is sensitive to interferences due to the presence of dissolved organic and inorganic compounds in the water that absorb at or near the 260 nm UV region (Gordon and Pacey 1986; Stanley and Johnson, 1986).

The direct UV method is good for studies in which the water is relatively clean (i.e. buffered DI water matrix or ozone stock solution) and for experimental control purposes, but is not sufficiently accurate for quantitative work, particularly when there is significant background absorbance (Eaton et al., 1999) as in the case of turbid pipe wash water.

The ozone concentration from $A_{260}$ measurement were determined by multiplying the $A_{260}$ measurement with a factor of 14.54, based on a molar absorptivity of 3,300 $M^{-1}$ $cm^{-1}$ for ozone. An integrated average ozone Ct was calculated based on the indigo colorimetric measurements. The integrated average ozone Ct was calculated using the area under curve (or trapezoidal) method. The remaining ozone at the end of the prescribed contact time was neutralized by adding sterilized 1.0 *M* sodium formate.

### *ii. Free Chlorine Experiments*

Free chlorine stock solution was prepared by diluting purified sodium hypochlorite solution (4-6% NaOCl, BDH Inc., England) into ODF water to give a concentration in the range of 150 to 300 mg/L, based upon the free chlorine dose required. The concentration of diluted solution was measured by N, N-diethyl-p-phenylenediamine (DPD) standard colorimetric method using an Ultraspec 2000 spectrophotometer at $A_{515}$ and a 1 cm path length cuvette.

The unscraped coupon was suspended in 100 to 500 mL of sterilized ODF phosphate buffer in 500 or 1000 mL reactor flasks, covered with aluminum foil. The phosphate buffer (in the reactor flask) was stirred gently to provide mixing of free chlorine with the phosphate buffer but to minimize loss of free chlorine from the reactor solution. An aliquot of freshly prepared chlorine stock solution was added to the reactor to provide a target free chlorine residual of 25 mg/L at the end of the contact time. This was consistent with the target free chlorine residual used by Denver Water during the water main disinfection, according to AWWA C651 standard.

Free chlorine concentration in the reactor flask solution was measured at various times during the contact time (by extracting the samples from the reactor flask) using the DPD

colorimetric method. An integrated average free chlorine Ct was calculated based on these measurements. The remaining free chlorine at the end of the prescribed contact time was neutralized by adding sterilized 0.1 *N* sodium thiosulfate.

*iii. Enumeration of HPC Bacteria Remaining on the Biofilm*

Following neutralization of the disinfectant (ozone and free chlorine), the biofilm coupon was removed from the reactor flask. The solution remaining in the reactor was homogenized (for 3 minutes at 25,000 revolutions per minute using a laboratory tissue homogenizer) and a sample was extracted and enumerated for HPC bacteria to provide a measure of the survival of HPC bacteria that sloughed from the biofilm during the contact time.

The coupon was then scraped with a rubber policeman and the biofilm was collected in the remaining phosphate buffer in the reactor flask. This solution was homogenized for 3 minutes at 25,000 revolutions per minute. The homogenized solution was enumerated for HPC bacteria to provide a composite measurement of HPC bacteria in the biofilm and in sloughed material. The concentration of HPC bacteria remaining in the biofilm, attached to CML coupon surface, was determined by the difference between the composite bacteria measurement and the sloughed bacteria measurement.

The maximum elapsed time between sample collection and HPC bacteria enumeration was usually about 45 min. Unscraped samples that could not be analyzed within one hour were maintained at a temperature of 4°C for a maximum of 24 hours.

### 3.4.3 Disinfection of Suspended Bacteria

*i. Preparation of samples*

In initial experiments on suspended HPC bacteria in the pipe wash water it was found that the initial demand for ozone and free chlorine was very high. Therefore, 200 mL of pipe wash water was settled for 2 minutes in sterilized 250 mL glass beaker and the supernatant was diluted with ODF phosphate buffer at a ratio of 1:100 for ozone experiments and 1:10 for free chlorine experiments in an experimental reactor flask. The

diluted contents in the reactor flask were mixed thoroughly for 2 minutes, using a magnetic stirrer. Prior to addition of disinfectant a sample was taken for initial HPC bacterial concentration.

### *ii. Ozone Experiments*

The ozone disinfection experiments for suspended bacteria were carried out in either 500 mL or 1000 mL reactor flasks, containing 200 to 300 mL diluted pipe wash water. These experiments were run in randomized order in terms of contact time. The purpose of randomization was to reduce the effect of nuisance variables on the interpretation of the disinfection results.

The ozone stock solution was metered into the reactor flask using a peristaltic pump at a rate sufficient to maintain the ozone residual between 0.5 and 1 mg/L. The ozone delivery rate (target ozone residual) in the solution in the reactor flask was monitored in real-time by re-circulating a portion of the reactor flask solution through a 35 $\mu$L, 10 mm path length flow cell, measuring $A_{260}$ on the Hewlett-Packard (model 8452A) diode-array spectrophotometer. The $A_{260}$ measurements were supplemented by periodically extracting samples for ozone measurement using the indigo colorimetric method ($A_{600}$) with a 1 cm path length cuvette on Ultra spec 2000 spectrophotometer.

The integrated average ozone Ct was calculated by the trapezoidal method. The remaining ozone at the end of the prescribed contact time was neutralized by adding sterilized 1.0 *M* sodium formate.

### *iii. Free Chlorine Experiments*

The free chlorine disinfection experiments for suspended bacteria were carried out in 250 mL sterilized ODF reactor flasks containing diluted pipe wash water. A sample of diluted pipe wash water, prior to disinfection, was enumerated for initial HPC bacteria concentration.

These experiments were also run in randomized order in terms of contact time. An aliquot of free chlorine stock solution was added to the reactor flask at time equal to zero. The size of the free chlorine aliquot was such that the final chlorine residual at the end of the contact time was not less than 25 mg/L. This was consistent with the target free chlorine residual used by the Denver Water during their water main disinfection, according to AWWA C651 standard.

The free chlorine concentration in the reactor flask was measured at various times (during the contact time) by extracting a sample and measuring the free chlorine concentration in the sample using the DPD colorimetric method.

An integrated average free chlorine Ct was calculated based on the DPD colorimetric measurements. The remaining free chlorine at the end of the prescribed contact time was neutralized by adding sterilized 0.1 *N* sodium thiosulfate.

# CHAPTER 4: RESULTS AND DISCUSSION

## INTRODUCTION

This chapter is organized into five main sections. The first section deals with the biofilm annular reactor operation including monitoring of reactor water for growth of biofilms and determination of HPC bacteria concentration in laboratory-grown biofilms. The second section covers biofilm HPC bacteria inactivation results, using ozone and free chlorine as disinfectants, and comparison of these results. The third section describes suspended HPC bacteria inactivation results using ozone and free chlorine, and comparison of these results. This section also covers a discussion on biofilm and suspended HPC bacteria disinfection results using ozone and free chlorine, respectively. In the fourth section the inactivation results are compared with the available literature and a concluding note is made on the significance of these results for field applications. The final section talks about experimental limitations of this study.

## 4.1 BIOFILM ANNULAR REACTOR OPERATION

It is earlier stated that, the main objective of this study was to determine the effectiveness of ozone, as an alternative disinfectant, for inactivation of biofilms under field conditions. The field conditions such as target disinfectant residual, exposure (contact) time and pH were specified by Denver Water.

For biofilm disinfection experiments, biofilms were grown on CML coupons in an annular reactor (also called as biofilm reactor in this study) using pipe wash water that was used for washing and cleaning of water mains of Denver Water. This was to ensure that biofilm on coupons was composed of the same mix of naturally occurring microorganisms. The resulting biofilm, however, was probably not identical to the biofilm that grows on the water mains in the pipe yard of the Denver Water. To ensure the availability of organic substrate in (recirculated) pipe wash water for growth of biofilms, it was necessary to monitor the water in the biofilm reactor. COD and TOC tests were, therefore, used as the standard measurements. Additional details of biofilm reactor operation can be found in appendix "B."

### 4.1.1 COD

The COD of the water in the biofilm reactor was measured daily from March 3 to July 19 (Figure 4-1) by collecting water samples from an external reservoir (Figure 4-1 and Photo 3-2). The purpose was to ensure the availability of organic substrate in the water for biofilm growth.

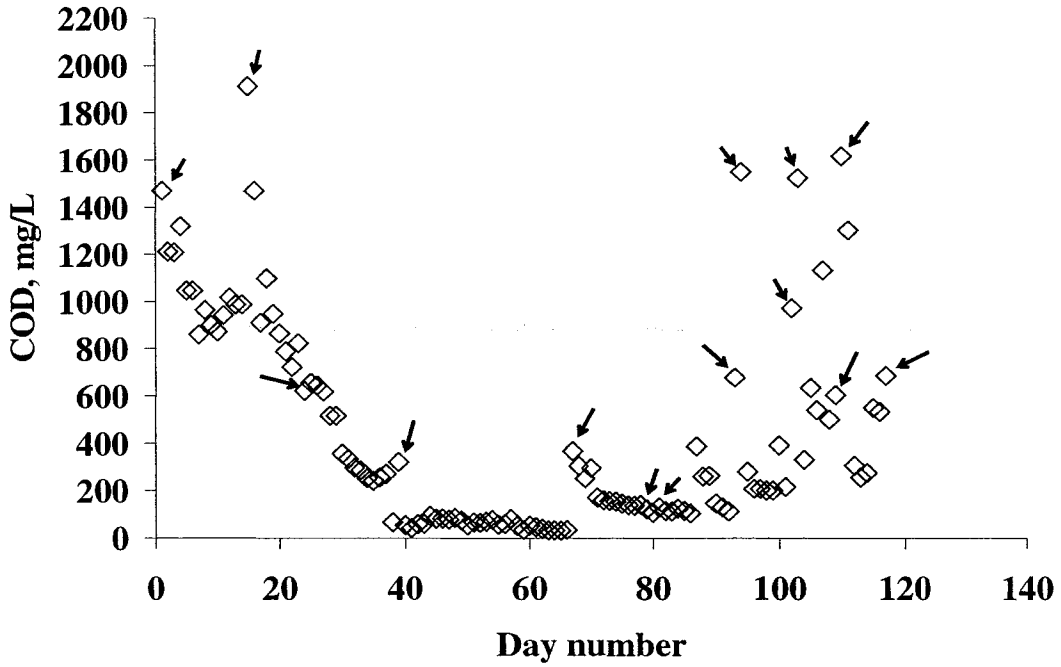


**Figure 4-1:** COD monitoring of biofilm reactor water (March 3 - July 19, 2004) (Arrows indicate days on which fresh water was added to the reservoir for recirculation through biofilm reactor)

Figure 4-1 indicates the COD of biofilm reactor water was in the range of 31 to 1,470 mg/L, excluding the single extreme value of 1,912 mg/L (March 16) as an outlier. The COD was high at the time of adding fresh pipe wash water to external reservoir but decreased with time. The decrease in the COD was presumed to be due to consumption of organic substrate by bacteria for their multiplication and growth on CML coupons. The variability in COD values, on the day of fresh water addition, is due to the variability of pipe wash water samples that were received periodically from Denver Water.

### 4.1.2 TOC

The COD test was supplemented with the TOC test in order to measure the fraction of organic carbon in the biofilm reactor water that cannot be measured by the COD test. The TOC of biofilm reactor water was measured daily from March 3 to May 7 as shown in Figure 4-2. The TOC could not be monitored beyond May 7 because of operational problems with the TOC analyzer.

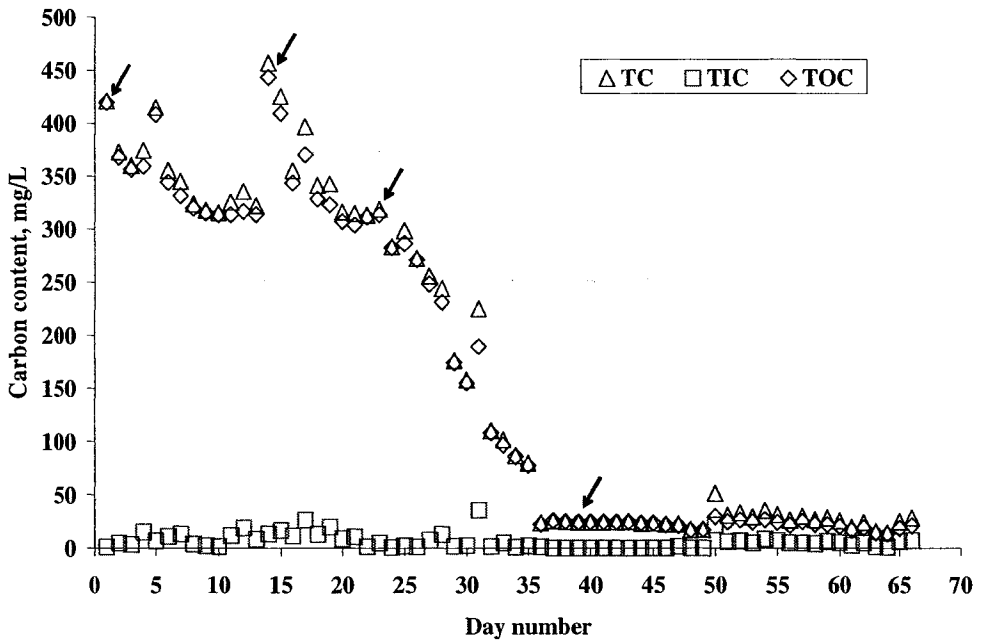


**Figure 4-2:** Carbon monitoring of biofilm reactor water (March 3 - May 7, 2004) (Arrows indicate days on which fresh water was added to the reservoir for recirculation through biofilm reactor)

Figure 4-2 shows the TOC of biofilm reactor water was in the range of 13 to 450 mg/L. Similar to COD of biofilm reactor water (Figure 4-1) the TOC was high at the time of adding fresh biofilm reactor water to the external reservoir but decreased with time. The decrease in the TOC was presumed to be due to consumption of organic substrate by HPC bacteria for their multiplication and growth on the CML coupons. Figure 4-2 indicates that over 99% of the total carbon (TC), in biofilm reactor water, was in the form of organic carbon (TOC). This may have caused a high initial demand for disinfectants (ozone and free chlorine) during the disinfection experiments.

### 4.1.3 Biofilm Age/Growth Period

Biofilms of various ages (length of the growth period in an annular reactor, in weeks), as required by Denver Water, were used for disinfection experiments with ozone and free chlorine. The concentration of HPC bacteria ($CFU/cm^2$) of twenty five (25) biofilm samples, used in this study, is plotted against biofilm age (Figure 4-3).

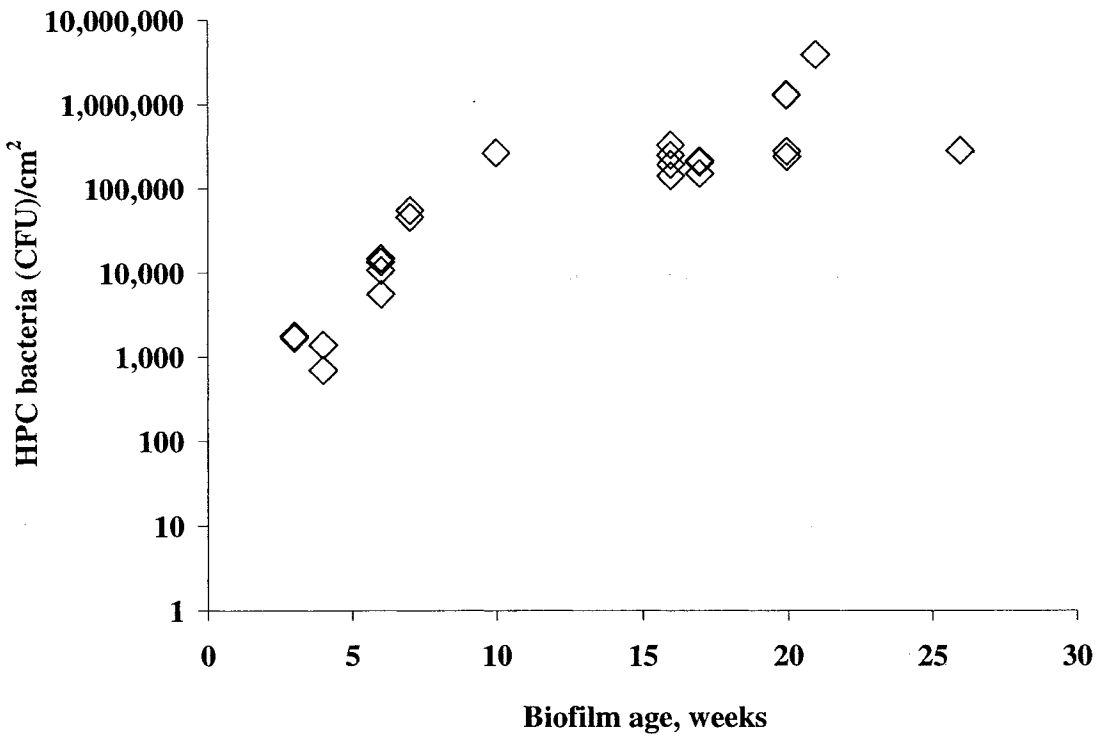


**Figure 4-3:** Concentration of biofilm HPC bacteria corresponding to biofilm age

Figure 4-3 indicates that the concentration of biofilm HPC bacteria increased initially with biofilm age (or growth period), but it eventually stabilized at $10^5$ to $10^6$ $CFU/cm^2$ after about 10 weeks. This shows that the biofilm HPC bacteria concentration is a function of biofilm age. A biofilm concentration in this range ($10^5$ to $10^6$ $CFU/cm^2$) was sufficient for measurement of more than 5 log inactivation.

In early biofilm disinfection experiments with ozone and free chlorine, the biofilm age ranged from 10 to 17 weeks and 20 to 21 weeks, respectively. This growth period resulted in the establishment of a relatively thick biofilm on CML coupon that could be observed with naked eye (Photo 4-1). Biofilm growth appeared non-uniform and "patchy". For the purpose of this study, biofilms that were between 16 and 21 weeks of age were arbitrarily designated as "mature" or "thick' biofilms. Biofilms that were 3 to 7 weeks old were arbitrarily designated as "immature" or "thin" biofilms (Photo 4-2).

**Photo 4-1:** A sample of mature or thick biofilm grown on CML coupon in a biofilm reactor at room temperature

**Photo 4-2:** A sample of immature or thin biofilm grown on CML coupon in a biofilm reactor at room temperature

It should be mentioned over here, such arbitrary designations to describe biofilms are also used by other researchers. For example, Chen and Stewart (1996) described their artificial biofilms of *Pseudomonas aeruginosa* as "thin" (428 μm) and "thick" (526 μm) biofilms. Similarly, Wood et al. (1998) used the designations "thin" and "thick" *Pseudomonas aeruginosa* biofilms (30 to 100 μm biofilms as thick biofilms). Also, Morton et al. (1998) have reported the use of these designations for biofilms. Bishop and Yu (1999) considered their two months old biofilms (aerobic/sulfate-reducing and aerobic/nitrifying biofilms) as "mature" biofilms.

## 4.2 BIOFILM DISINFECTION

In order to investigate ozone as an alternative disinfectant for biofilm inactivation, a number of experiments were conducted using biofilm samples that were grown in the laboratory, at the room temperature. The disinfection (inactivation) results are reported as log inactivation of biofilm HPC bacteria (at corresponding Ct values). The biofilm disinfection results were compared with those of free chlorine to determine the relative effectiveness of ozone for biofilm inactivation.

### 4.2.1 Ozone Disinfection of Biofilms

Table 4-1 summarizes the results of biofilm inactivation using ozone as a disinfectant. All of the experiments, with mature biofilm, were randomized to reduce the effect of nuisance variables (such as non-uniform biofilm growth) on the interpretation of disinfection results.

**Table 4-1:** Results summary of ozone disinfection experiments of biofilm HPC bacteria suspended in 0.05 M ODF phosphate buffer at 22°C

| Run No. | biofilm age | pH | contact time, t | applied ozone dose | avg. ozone residual | Ct | initial HPC in biofilm | final HPC | | Log inactivation | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | biofilm+ sloughed | sloughed | including sloughed HPC | based on biofilm HPC only |
| | wks | | min | mg/L | mg/L | mg x min/L | CFU/ $cm^2$ | CFU/ $cm^2$ | CFU/$cm^2$ | log-units | log-units |
| 1 | 16 | 6.9 | 60 | 9.7 | 0.42 | 25.2 | 142,000 | 15,600 | 130 | -0.96 | -0.96 |
| 2 | 16 | 6.9 | 5 | 3.3 | 1.18 | 5.9 | 194,000 | 49,600 | 59 | -0.59 | -0.59 |
| 3 | 17 | 6.9 | 8 | 4.7 | 0.40 | 3.2 | 206,000 | 64,200 | 61 | -0.51 | -0.51 |
| 4 | 17 | 6.9 | 16 | 4.4 | 0.65 | 10.4 | 215,000 | 92,800 | 134 | -0.36 | -0.36 |
| 5 | 17 | 6.9 | 40 | 15.1 | 0.58 | 23.2 | 151,000 | 12,200 | 26,100 | -1.09 | -0.60 |
| 6 | 17 | 6.9 | 70.5 | 17.0 | 0.40 | 28.2 | 215,000 | 3,800 | 19,700 | -1.75 | -0.96 |
| 7 | 16 | 6.9 | 62 | 13.4 | 0.91 | 56.3 | 332,000 | 31,800 | 3,600 | -1.02 | -0.97 |
| 8 | 26 | 6.9 | 12 | 3.7 | 0.69 | 8.3 | 283,000 | 32,300 | 1,720 | -0.94 | -0.92 |
| 9 | 10 | 6.9 | 7 | 2.3 | 0.70 | 4.9 | 267,000 | 13,900 | 859 | -1.28 | -1.26 |
| 10 | 16 | 6.9 | 32 | 10.7 | 0.69 | 22.1 | 250,000 | 32,600 | 1,810 | -0.88 | -0.86 |
| 11 | 3 | 6.0 | 8 | 2.6 | 0.21 | 1.7 | 1,800 | 60,600 | 0 | 1.53 | 1.53 |
| 12 | 3 | 6.0 | 11 | 2.9 | 0.38 | 4.2 | 1,700 | 26,700 | 0 | 1.20 | 1.20 |
| 13 | 4 | 6.0 | 65 | 3.6 | 0.70 | 45.5 | 1,400 | 800 | 0 | -0.24 | -0.24 |
| 14 | 4 | 6.0 | 63 | 3.5 | 0.83 | 52.3 | 700 | 1,000 | 0 | 0.15 | 0.15 |

All fourteen experiments (or runs) were conducted by metering aqueous ozone stock solution into reactor flasks, with an ozone residual target of 0.5 to 1.0 mg/L, except Runs 1 and 2 (Table 4-1). These two experiments were conducted with a spike of ozone addition followed by metered addition of ozone stock solution. The spiked addition was eliminated in later experiments because it is not representative of field conditions.

Experiments 1 to 10 for mature biofilms were conducted using pH 6.9 phosphate buffer whereas the remaining (immature biofilm) experiments were conducted using pH 6.0 phosphate buffer. It should be noted that one of the challenges in the first set of ozone disinfection experiments (Runs 1 to 10) was achieving the ozone residual target, due to high ozone demand and rapid decay. The pH of phosphate buffer was reduced to 6.0 in later experiments (Runs 11 to 14 for immature biofilms) in order to stabilize the ozone in the reactor flask solution. The reduction in pH of phosphate buffer, however, does not affect disinfection efficiency (U.S. EPA, 1999).

In Table 4-1, biofilm age is the length of growth period in an annular reactor. Contact time (t) represents the holding time of ozone in reactor flask solution. Applied ozone dose is the mass (volume) of ozone delivered from the ozone stock solution divided by final volume of solution in the reactor flask. Average ozone residual (ozone concentration in the reactor flask) is weighted average of consecutive measurements based on the indigo colorimetric method. Each measurement was obtained by the difference between final baseline absorbance at 600 nm (following sodium formate addition) and absorbance measured at a given contact time (Gyüre'k and Finch, 1998). This measurement represents 'C' of Ct value.

Initial HPC bacteria concentration ($CFU/cm^2$) represents the concentration of biofilm HPC bacteria on a CML coupon that was not exposed to ozone. Final HPC bacteria concentration in a biofilm was determined by multiplying the average number of viable cells (CFU/mL) with the final reactor flask volume (mL) and then dividing the product with total surface area ($cm^2$) of exposed coupon.

Final sloughed HPC bacteria concentration represents the HPC bacteria concentration in the reactor flask volume prior to scraping of ozonated biofilm. Sloughed bacteria are the bacteria that sloughed spontaneously from the coupon during the ozone exposure period and that survived ozone exposure in the bulk solution. Final biofilm and sloughed HPC bacteria concentration represent HPC bacteria concentration in the reactor flask volume after scraping the biofilm, at the end of contact time.

Figure 4-4a and Figure 4-4b show examples of ozone residual profiles, determined by direct UV ($A_{260}$) method.

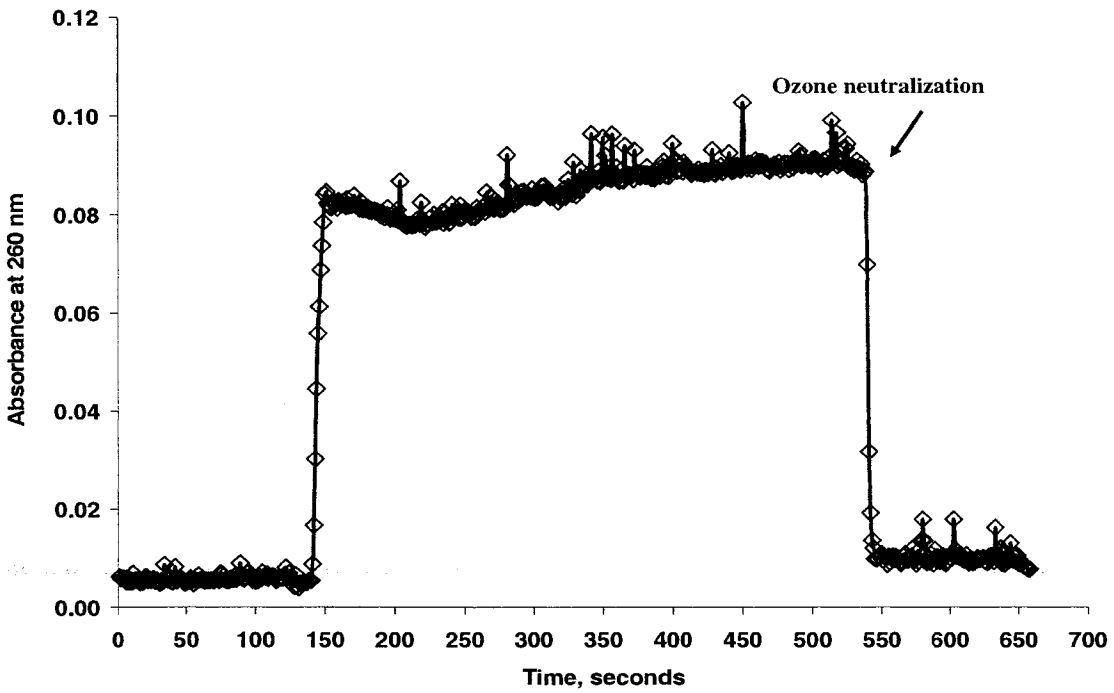


**Figure 4-4a:** Ozone residual profile for 5-min disinfection experiment (Run 2, Table 4-1) (spike of ozone stock solution addition followed by metered addition)

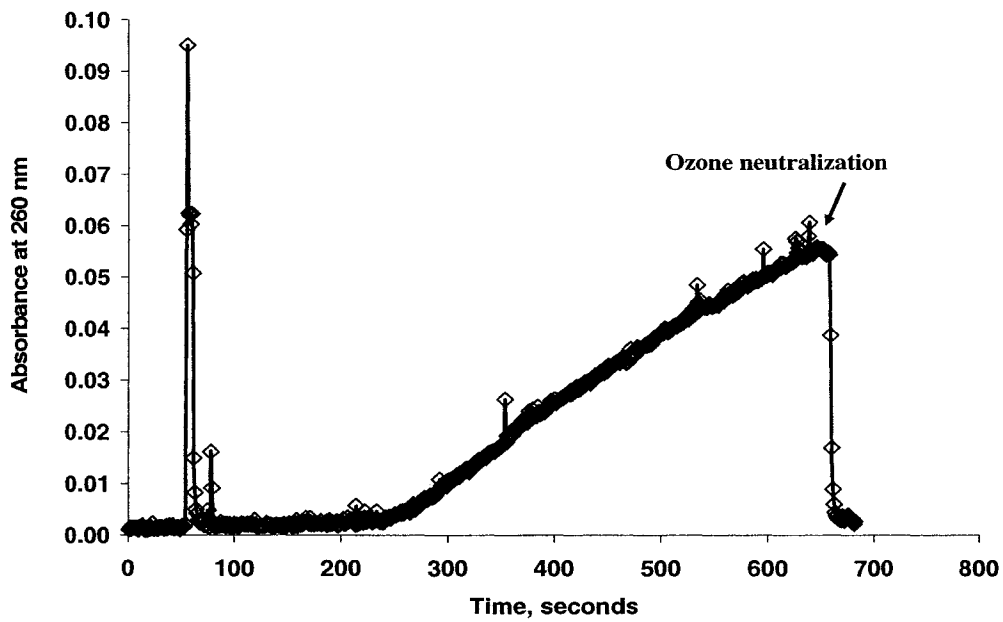


**Figure 4-4b:** Ozone residual profile for 5-min disinfection experiment (Run 3, Table 4-1) (metered addition of ozone stock solution only)

Figure 4-4a shows an ozone residual profile for 5-min disinfection experiment that was conducted by exposing the reactor flask volume (containing biofilm coupon) to a spike dose of ozone stock solution, followed by metered addition of ozone stock solution. The spike dose of ozone resulted in an instantaneous high ozone concentration in the reactor flask solution. At the end of contact time the reactor flask solution was neutralized by adding 1.0 M sodium formate.

Figure 4-4b shows an ozone residual profile for 5-min disinfection experiment with only metered addition of ozone stock solution. The initial spikes at ~ 60 to 70 seconds were due to temporary removal of the ozone delivery tube from the reactor flask, to remove air bubbles in the tube.

The ozone disinfection results of biofilm HPC bacteria corresponding to average ozone Ct values are presented in Figure 4-5.

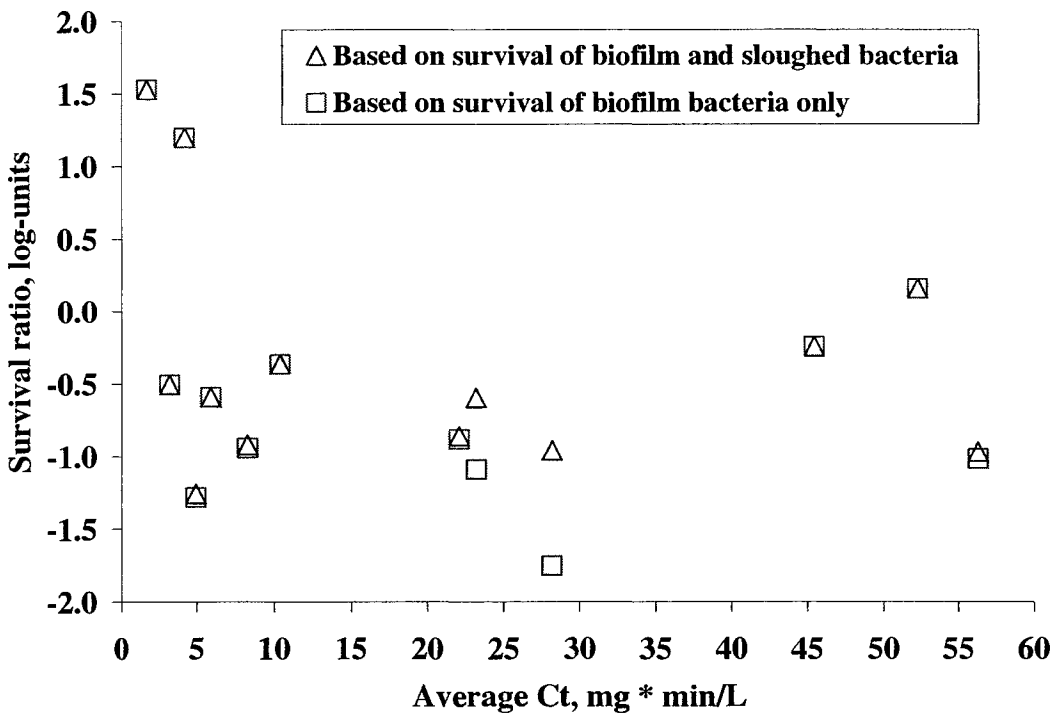


**Figure 4-5:** Log inactivation of biofilm HPC bacteria versus average ozone Ct value at 22 °C

Figure 4-5 shows no difference in log inactivation of both sloughed biofilm and non-sloughed biofilm (biofilm attached to the CML coupon) HPC bacteria, for most of the experiments. In other words, when sloughed bacteria were included in the log inactivation calculations, the inactivation was approximately the same as when the calculation was based on biofilm bacteria only. This indicates that little sloughing occurred during the experiment or if the biofilm sloughed, it (biofilm HPC bacteria) did not survive on exposure to ozone.

This should be mentioned over here that, sloughed bacteria are not as likely to be of health concern in the field application because the water mains are usually flushed thoroughly prior to bacterial testing and putting into service (AWWA, 1999).

Figure 4-5 shows that the log inactivation of biofilms was in the range of +1.5 to -1.8 for Ct range of 1.7 to 56.3 mg x min/L. The statistical (regression) analysis of this data set (14 Runs) shows $R^2$ value of only 0.0569 at 95% confidence interval. This indicates almost no relationship between Ct value and log inactivation of biofilm HPC bacteria, for the conditions tested. This may be due to biofilm inactivation results at different experimental conditions.

It should be noted that Runs 1 to 10 were conducted with mature biofilms, and Runs 11 to 14 were conducted with immature biofilms. Therefore, to determine statistical relationship between Ct values and log inactivation (or log inactivation of biofilms as a function of ozone), the ozone disinfection results for mature biofilms (Figure 4-6) and immature biofilms (Figure 4-7) were analyzed separately.

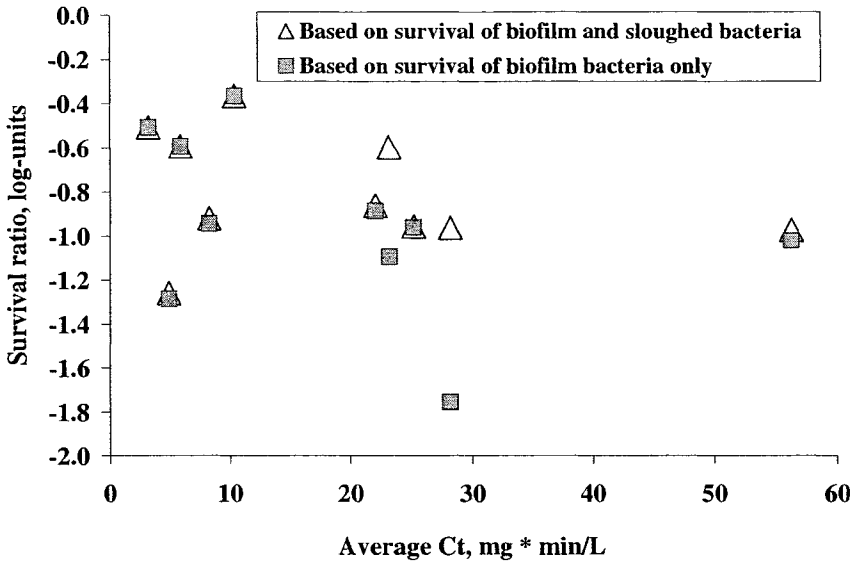


**Figure 4-6:** Log inactivation of mature biofilm HPC bacteria versus average ozone Ct value at 22°C (Runs 1 to 10; pH 6.9)

Figure 4-6 shows that the log inactivation of mature biofilm HPC bacteria by ozone was generally limited to about 1 log-unit for (ozone) Ct range of 3.2 to 56.3 mg x min/L (Runs 1 to 10 at pH 6.9).

Performing regression analysis with 95% confidence interval for this data set (Runs 1 to 10), based on log inactivation of biofilm HPC bacteria only, it was found that $R^2$ was only 0.1482 and *P*-values for intercept and slope were 0.0047 and 0.2719, respectively. Considering log inactivation of -1.75 at 28.2 mg x min/L (Run 6) as an outlier, no significant change was observed in $R^2$ and *P*-values. Since the *P*-value for slope is greater than 0.05, it can be concluded that the slope is insignificant at 95% confidence interval. In other words, based upon $R^2$ of only 0.1482 and *P*-value for slope greater than 0.05, there is not a statistical linear relationship between Ct value and log inactivation of biofilm HPC bacteria at the 95% confidence level. This suggests that, for the conditions tested, mature biofilm inactivation is not a function of ozone Ct value. If this was the case, an increase in ozone Ct value would result in only limited additional inactivation of biofilm HPC bacteria. However, further experiments would need to be conducted to confirm this such as more data at large Ct values.

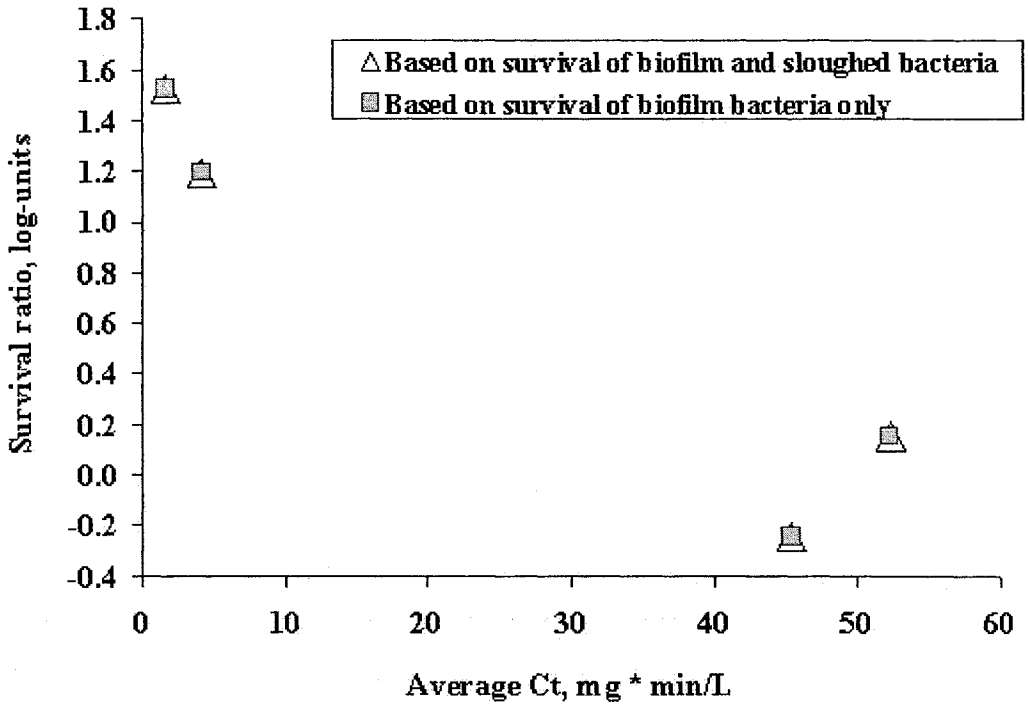


**Figure 4-7:** Log inactivation of immature biofilm HPC bacteria versus average ozone Ct values at 22°C (Runs 11 to 14; pH 6.0)

Table 4-1 shows that little sloughing in all four experiments (Runs 11 to 14 at pH 6.0) occurred or if the biofilm HPC bacteria sloughed, it (biofilm HPC bacteria) did not survive on exposure to ozone. The regression analysis for this data set (Runs 11 to 14 at ozone Ct range of 1.7 to 52.3 mg x min/L) produced $R^2$ of 0.8984 and *P*-values of 0.0283 and 0.0521 for intercept and slope, respectively with 95% confidence interval. Although, the *P*-value for slope is slightly greater than 0.05 (at 95% confidence interval), the dataset suggests that there may be a statistical linear relationship between Ct value and log inactivation of immature biofilm HPC bacteria. In other words, for the conditions tested, immature biofilm inactivation may have been a function of ozone Ct value. This means, the increased ozone Ct value may have resulted in increased log inactivation of immature biofilm HPC bacteria. It is difficult to conclude because the data set is limited i.e. only 4 runs were conducted for immature biofilm inactivation by ozone. It would be valuable to conduct further experiments using immature biofilms in order to confirm this conclusion.

The disinfection results of immature biofilm HPC bacteria (Figure 4-7) indicate negative inactivation of biofilm bacteria except one result of 0.24 log inactivation at Ct value of 45.5 mg x min/L (Run 13 in Table 4-1). Table 4-1 indicates that, under the conditions tested, the measured number of immature biofilm HPC bacteria actually increased following the exposure to ozone. The potential reason for negative inactivation could be the nature of immature biofilm growth (biofilm morphology). It may be possible that the immature biofilm HPC bacteria became weak once exposed to ozone due to reaction between ozone and biofilm EPS. In other words, ozone may have reacted with the immature biofilm EPS without necessarily inactivating the biofilm HPC bacteria. Therefore, upon homogenization, the biofilm HPC bacteria were dispersed as discrete cells thus increasing the number of colonies counted ($N_t$). Whereas, in the case of unozonated biofilm samples, the homogenization process could not disperse the bacterial clusters as thoroughly and the assumption of 1 CFU as viable 1 cell was not satisfied (Figure 4-8).

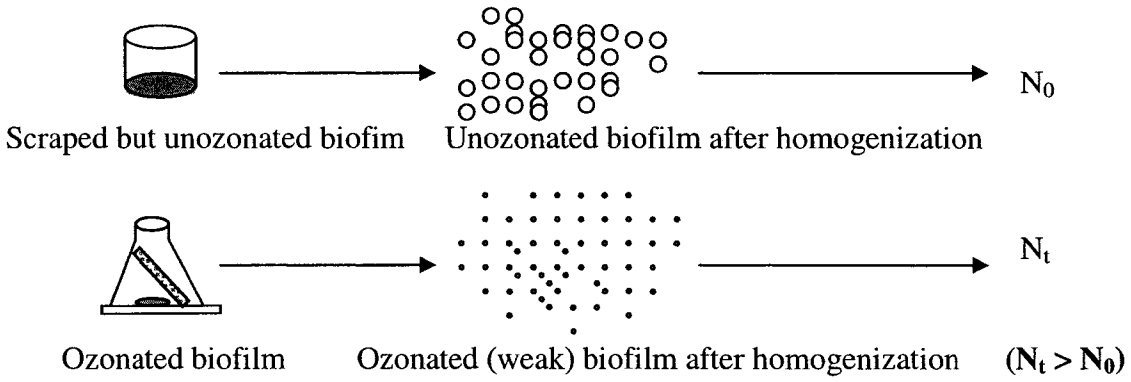


**Figure 4-8:** Diagrammatic representation of possible reason for negative inactivation of immature biofilm bacteria exposed to ozone (Runs 11 to 14; pH 6.0)

### 4.2.2 Free Chlorine Disinfection of Biofilms

Table 4-2 summarizes the results of all free chlorine disinfection experiments for biofilm HPC bacteria. These experiments were conducted in a manner similar to ozone disinfection experiments for biofilms, except that they were exposed to free chlorine.

**Table 4-2:** Results summary of free chlorine disinfection experiments of biofilm HPC bacteria suspended in 0.05 M ODF phosphate buffer at 22°C

| Run No. | biofilm age | contact time, t | applied chlorine dose | avg. chlorine residual | Ct | initial HPC in biofilm | final HPC | | Log inactivation | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | biofilm + sloughed | sloughed | including sloughed HPC | based on biofilm HPC only |
| | weeks | min | mg/L | mg/L | mg x min/L | CFU/ $cm^2$ | CFU/ $cm^2$ | CFU/ $cm^2$ | log-units | log-units |
| 1 | 20 | 60 | 31.94 | 28.50 | 1,710 | 277,000 | 6,960 | 870 | -1.60 | -1.55 |
| 2 | 20 | 240 | 40.40 | 34.60 | 8,304 | 1,310,000 | 53 | 0 | -4.39 | -4.39 |
| 3 | 20 | 480 | 35.47 | 27.40 | 13,152 | 238,000 | 52 | 0 | -3.66 | -3.66 |
| 4 | 20 | 720 | 42.00 | 31.40 | 22,608 | 1,280,000 | 54 | 0 | -4.37 | -4.37 |
| 5 | 21 | 1440 | 40.09 | 28.70 | 41,328 | 3,920,000 | 0 | 0 | >-6.59 | >-6.59 |
| 6 | 6 | 5 | 26.66 | 26.59 | 133 | 5,600 | 73,912 | 0 | 1.12 | 1.12 |
| 7 | 6 | 60 | 31.30 | 30.61 | 1,837 | 10,800 | 183 | 0 | -1.77 | -1.77 |
| 8 | 6 | 1440 | 39.99 | 36.15 | 52,056 | 13,600 | 0 | 0 | -4.13 | -4.13 |
| 9 | 7 | 60 | 27.85 | 24.08 | 1,445 | 55,100 | 587 | 592 | -1.97 | -1.67 |
| 10 | 6 | 5 | 23.99 | 22.40 | 112 | 15,000 | 1,159 | 1,800 | -1.11 | -0.70 |
| 11 | 7 | 1440 | 31.58 | 28.40 | 40,896 | 46,000 | 1 | 28 | -4.66 | -3.20 |

Runs 1 to 5 – at pH 6.9 Runs 6-11 – at pH 9.0

The experiments 1 to 5 (Table 4-2) were conducted in pH 6.9 phosphate buffer and experiments 6 to 11 were conducted in pH 9.0 buffer. The pH 9.0 buffer was used to reflect the pH of superchlorinated water in the water mains following holding time of disinfectant at Denver Water.

In Table 4-2, applied chlorine dose is the mass of free chlorine delivered from chlorine stock solution divided by final volume of solution in the reactor flask. Average chlorine residual (free chlorine concentration in the reactor flask) is weighted average of consecutive free chlorine concentration measurements based on the DPD colorimetric method. This value represents 'C' of Ct value.

The chlorine disinfection results of biofilm samples (log inactivation of HPC bacteria in biofilms) corresponding to average free chlorine Ct values are presented in Figure 4-9.

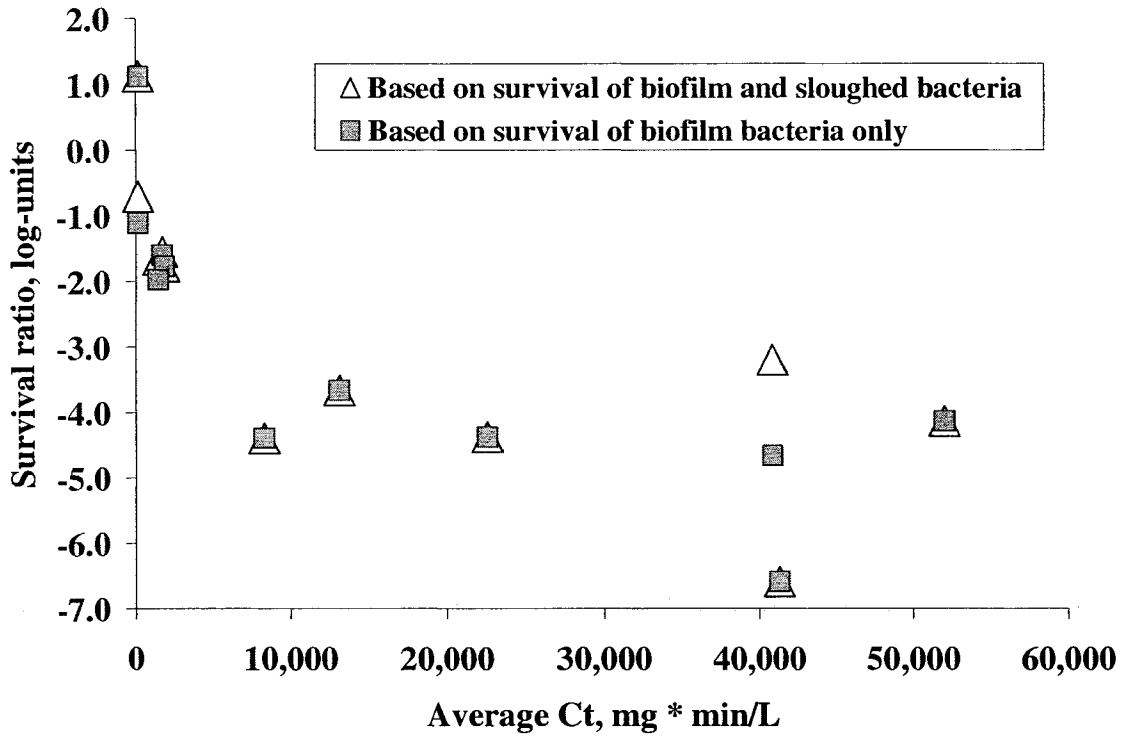


**Figure 4-9:** Log inactivation of biofilm HPC bacteria versus average free chlorine Ct value at 22°C

Figure 4-9 and Table 4-2 show no difference in log inactivation when sloughed biofilm HPC bacteria were accounted for compared to when only attached biofilm HPC bacteria were measured, except Run 10 (Table 4-2). This indicates that little sloughing occurred or if it occurred, the sloughed HPC bacteria were inactivated easily by exposure to free chlorine. Similar observation was noted in ozone disinfection experiments for biofilm HPC bacteria (Figure 4-5).

Figure 4-9 shows that the log inactivation of biofilms was in the range of +1.12 to -4.39 for Ct range of 112 to 52,056 mg x min/L (Runs 1 to 11 in Table 4-2), excluding Run 5 result of over 6 log inactivation. The statistical (linear regression) analysis of this data set (14 Runs), at 95% confidence interval, shows $R^2$ value of 0.5745 and *P*-values of 0.0247 and 0.0069 for intercept and slope, respectively.

Based upon *P*-values for slope and intercept (less than 0.05) there seems a strong statistical relationship between free chlorine Ct value and log inactivation of biofilm HPC bacteria. However, $R^2$ value is not close to 1.0 indicating that the statistical relationship between biofilm HPC bacteria inactivation and free chlorine Ct value appears to be non-linear. Nevertheless, Figure 4-9 shows that biofilm bacteria inactivation is a function of free chlorine Ct value at lower free chlorine Ct values (i.e. < 10,000 mg × min/L). Because the exposure condition (free chlorine concentration) was constant during all the exposure period, inactivation results (Figure 4-9) suggests that some of HPC bacteria in biofilms were easily inactivated but others were resistant to free chlorine. These surviving bacteria may have sheltered from free chlorine exposure by diffusional limitation (Hall-Stoodley et al., 2004) or HPC is a mixed bacterial population (LeChevallier et al., 1987 reported HPC bacteria comprised of over 80 colony morphologies). Some species/strains may have greater resistance to free chlorine. It should also be noted that Runs 1 to 5 were conducted with mature biofilms, and Runs 6 to 14 were conducted with immature biofilms. This may have had some statistical effect on the disinfection results. Therefore, free chlorine disinfection results for mature biofilms (Figure 4-10) and immature biofilms (Figure 4-11) were analyzed separately.

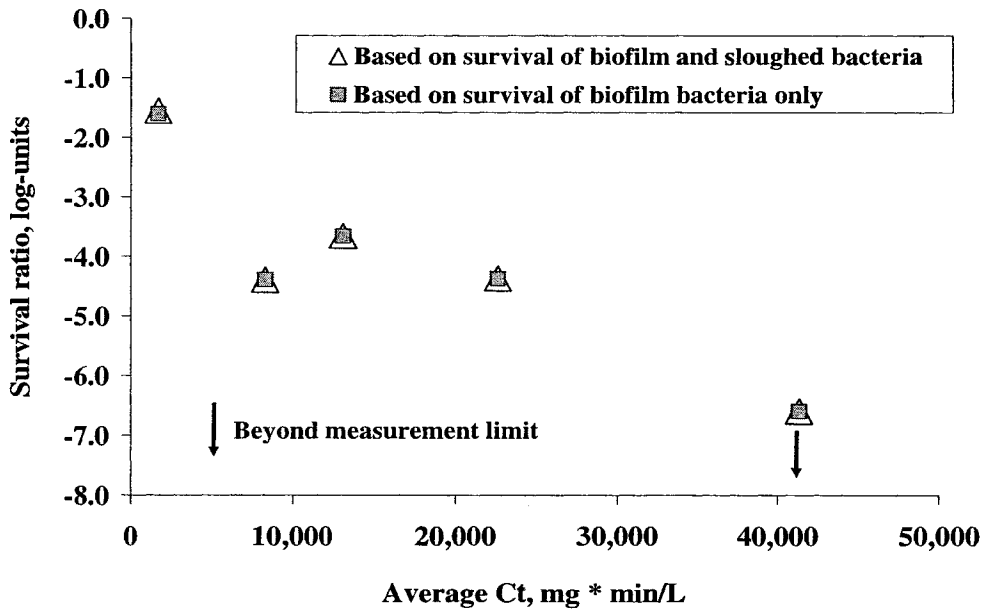


**Figure 4-10:** Log inactivation of mature biofilms versus average free chlorine Ct value at 22°C (Runs 1 to 5; pH 6.9)

Figure 4-10 shows log inactivation of 2 to beyond (or greater) than the measurement limit for mature biofilm HPC bacteria at free chlorine Ct range of 1,710 to 41,328 mg × min/L. Complete mature biofilm inactivation was observed at the highest free chlorine Ct value of 41,328 mg × min/L (Run 5 in Table 4-2). The high log inactivation of biofilm HPC bacteria using free chlorine (Figure 4-10) may be associated with the high experimental free chlorine Ct value. To verify this, regression analysis was performed for this data set (Runs 1 to 5), based on log inactivation of biofilm HPC bacteria.

The results showed $R^2$ of 0.8162 and *P*-values of 0.0371 and 0.0355 for intercept and slope, respectively. These values confirm a strong statistical linear relationship between free chlorine Ct value and log inactivation of mature biofilm HPC bacteria, under the conditions tested. Considering Run 5 value (over 6 log-units) at Ct of 41,328 mg × min/L as an outlier, much strong statistical relationship was found between free chlorine Ct value and log inactivation of mature biofilm HPC bacteria.

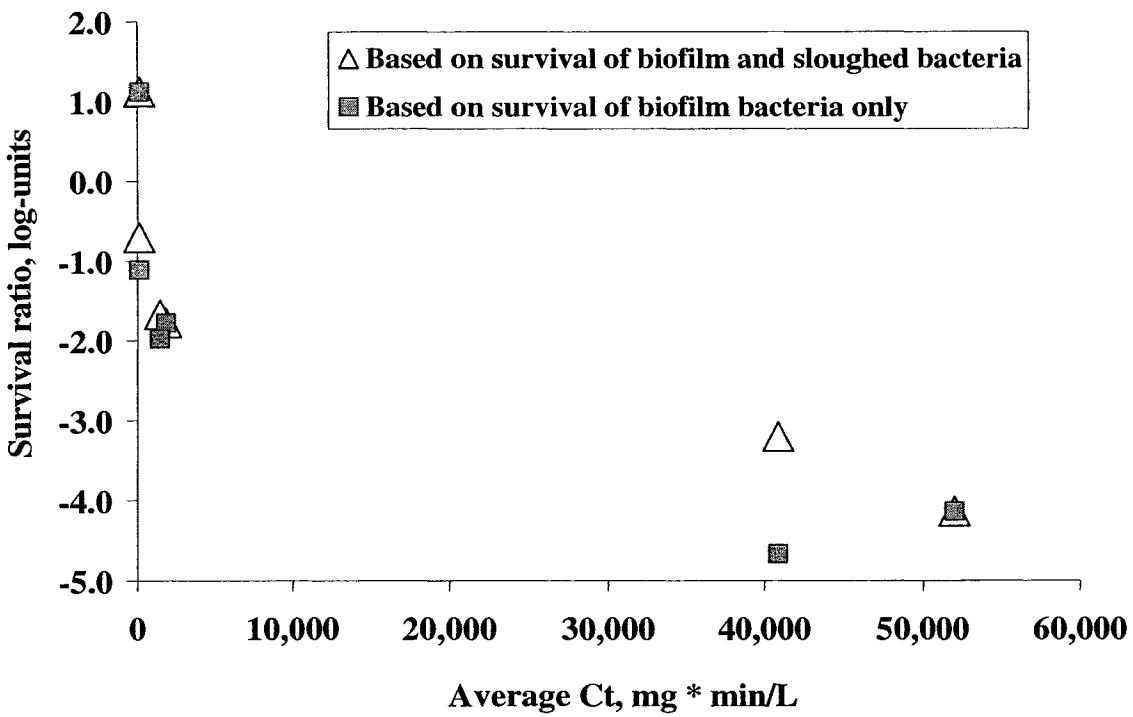


**Figure 4-11:** Log inactivation of immature biofilms versus average free chlorine Ct value 22°C (Runs 6 to 11; pH 9.0)

Similar to mature biofilm disinfection results (Figure 4-10), Figure 4-11 also shows an increase in log inactivation of immature biofilm HPC bacteria with an increase in free chlorine Ct value, at pH 9.0. Biofilm HPC bacteria inactivation of 1.1 to over 4 log-units was achieved at free chlorine Ct range of 112 to 52,056 mg × min/L, neglecting negative inactivation at Ct of 133 mg × min/L (Run 6 in Table 4-2).

The regression analysis for immature biofilms (Runs 6 to 11 in Table 4-2), based on log inactivation of biofilm HPC bacteria, shows $R^2$ of 0.7041 and *P*-values for intercept and slope as 0.2405 and 0.0367, respectively. Considering negative inactivation (+1.12) of Run 6 in Table 4-2 as an outlier, an $R^2$ of 0.8938 and *P*-values for intercept and slope of 0.0193 and 0.0152, respectively were obtained. The regression analysis results indicate a strong statistical linear relationship between Ct value and log inactivation of immature biofilm. In other words, biofilm disinfection is a function of free chlorine Ct value. This means, under the conditions tested, free chlorine disinfection results can be used for the disinfection of water mains in the field (Denver Water). However, it should be noted that the biofilms grown in the laboratory may not be representative of biofilms found on water mains of Denver Water.

Comparing the disinfection results of mature biofilm bacteria at pH 6.9 for Runs 1 to 5 (Figure 4-10) and immature biofilm bacteria at pH 9.0 for Runs 6-11 (Figure 4-11), not a significant difference in log inactivation was observed. At both pH conditions (pH 6.9 and pH 9.0), the biofilm HPC bacteria inactivation was up to 5 log-units, rejecting log inactivation of greater than the measurement limit at Ct value of 41,328 mg × min/L (Run 5 in Table 4-2) as an outlier. However, the free chlorine disinfection results show that, at low pH (pH 6.9), less free chlorine Ct value was required in comparison to similar log inactivation at high pH (pH 9.0). For example, at pH 6.9, 3.66 log inactivation of biofilm bacteria was achieved at a Ct value 13,152 mg × min/L (Run 3 in Table 4-2). In comparison, at pH 9.0, 3.20 log inactivation of biofilm bacteria was achieved at a Ct value of 40,896 mg × min/L (Run 11 in Table 4-2).

It should be noted that, pH 6.9 and pH 9.0 disinfection experiments were conducted on different biofilms, i.e. mature and immature biofilms. Therefore, it was not possible to conclude if this was a pH effect or biofilm age (or morphology) effect. In other words, pH was confounded with biofilm age. This was a limitation of the experimental study that arose due to practical constraint on availability of biofilm samples. In future experiments, it would be valuable to compare the effect of pH on biofilm of similar age. This is relevant because the pH of water in the field application may be as high as 9 when hypochlorite solution is used to disinfect new and repaired water main biofilms.

Comparing the disinfection results of biofilms using ozone (Figure 4-5) with those of free chlorine (Figure 4-9), it was clear that, for the conditions tested, the disinfection efficiency of ozone was significantly lower than that of free chlorine. However, this does not imply that ozone was not an effective biofilm disinfectant because Ct values used for free chlorine were much higher than those of ozone. The Ct values of ozone and free chlorine used in this study were chosen to reflect field conditions used by Denver Water. A direct comparison of ozone and free chlorine based on molar Ct values was not the objective of this study.

## 4.3 SUSPENDED BACTERIA DISINFECTION

In order to compare the disinfection effectiveness of ozone for biofilm HPC bacteria with that of suspended bacteria, a number of disinfection experiments were conducted on suspended bacteria using diluted pipe wash water. This section discusses the results of these experiments and also their comparison with biofilm HPC bacteria disinfection results.

### 4.3.1 Ozone Disinfection of Suspended HPC Bacteria

Table 4-3 summarizes the results of all ozone disinfection experiments for suspended HPC bacteria. The ozone concentration measurements are based on the indigo colorimetric method.

**Table 4-3:** Results summary of ozone experiments of suspended HPC bacteria using pipe wash water sample diluted (1:100) in 0.05 M phosphate buffer at 22°C

| Trial No. | pH | contact time, t | applied ozone dose | avg. ozone residual | Ct | initial HPC | final HPC | log inactivation |
|---|---|---|---|---|---|---|---|---|
| | | min | mg/L | mg/L | mg x min/L | in 1 mL | in 1 mL | log-units |
| 1 | 6.9 | 13 | 3.37 | 0.48 | 6.2 | 3,800 | 2 | -3.28 |
| 2 | 6.9 | 14 | 2.57 | 0.50 | 7.0 | 6,000 | 2 | -3.48 |
| 3 | 6.9 | 35 | 3.32 | 0.47 | 16.5 | 4,000 | 0 | >-3.60 |
| 4 | 6.9 | 66 | 4.11 | 0.28 | 18.5 | 4,000 | 0 | >-3.60 |
| 5 | 6.9 | 10 | 3.16 | 0.70 | 7.0 | 1,700 | 1 | -3.23 |
| 6 | 6.9 | 62 | 2.26 | 0.97 | 60.1 | 1,100 | 0 | >-3.04 |
| 7 | 6.9 | 12 | 2.41 | 0.88 | 10.6 | 1,500 | 0 | >-3.18 |
| 8 | 6.9 | 62 | 4.11 | 0.28 | 17.4 | 4,000 | 0 | >-3.60 |
| 9 | 6.9 | 33 | 2.29 | 0.98 | 32.3 | 850 | 0 | >-2.93 |
| 10 | 6.0 | 8 | 3.77 | 1.06 | 8.5 | 3,800 | 0 | >-3.58 |
| 11 | 6.0 | 7 | 2.93 | 1.14 | 8.0 | 5,000 | 0 | >-3.70 |
| 12 | 6.0 | 62 | 2.84 | 1.01 | 62.6 | 3,800 | 0 | >-3.58 |
| 13 | 6.0 | 62 | 7.53 | 1.00 | 62.0 | 2,500 | 0 | >-3.40 |

Table 4-4 shows the comparison of ozone results of this study for suspended HPC bacteria with similar results previously reported by Gyürék and Finch (1998). The comparative results are also presented in graphical form (Figure 4-12).

**Table 4-4:** Results summary of ozone experiments of suspended HPC bacteria in 0.05 M, pH 6.9 phosphate buffer at 22°C, reported by Gyürék and Finch (1998)

| Gyürek and Finch data (1998) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Trial No. | contact time, t | applied ozone dose | avg. ozone residual | Ct | initial HPC | final HPC | log inactivation |
| | min | mg/L | mg/L | mg x min/L | in 1 mL | in 1 mL | log-units |
| 1 | 9.75 | 0.84 | 0.47 | 4.58 | 3,740,000 | 6,360 | -2.8 |
| 2 | 0.58 | 2.05 | 1.90 | 1.10 | 6,860,000 | 36,800 | -2.3 |
| 3 | 14.78 | 1.19 | 0.68 | 10.05 | 5,280,000 | 705 | -3.9 |
| 4 | 14.93 | 0.33 | 0.19 | 2.84 | 5,350,000 | 6,130 | -2.9 |
| 5 | 9.87 | 0.31 | 0.21 | 2.07 | 2,290,000 | 6,350 | -2.6 |
| 6 | 4.67 | 0.53 | 0.46 | 2.15 | 2,550,000 | 6,450 | -2.6 |
| 7 | 9.8 | 1.00 | 0.82 | 8.04 | 4,330,000 | 4,060 | -3.0 |
| 8 | 9.53 | 2.13 | 1.69 | 16.11 | 3,960,000 | 479 | -3.9 |
| 9 | 1.0 | 0.52 | 0.46 | 0.46 | 58,300 | 638 | -2.0 |

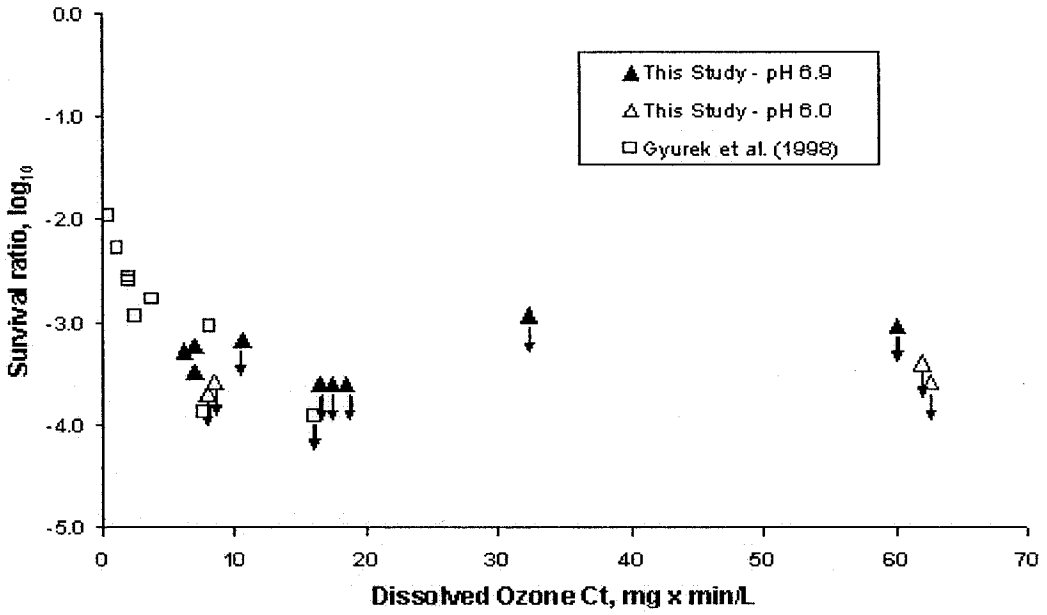


**Figure 4-12:** Log inactivation of suspended HPC bacteria versus average ozone Ct value at 22°C (pH 6.9). Experimental results from this study are compared to results of Gyürék and Finch (1998). ( ↓ Beyond measurement limit)

Figure 4-12 shows that the inactivation of suspended HPC bacteria by ozone was between 3 to 4 log-units or greater than measurement limit (Table 4-3) at both pH 6.0 and pH 6.9 conditions, as well as for all Ct values in the range of 6.2 to 62.6 mg × min/L. Figure 4-12 shows that ozone inactivation results of this study for suspended HPC bacteria (3 to 4 log-units at Ct range of 6.2 to 62.6 mg x min/L; Table 4-3) are consistent with the results reported by Gyürék and Finch (1998). Gyürék and Finch (1998) reported 2 to 4 log inactivation of suspended HPC bacteria by ozone at Ct range of 0.46 to 16.11 mg x min/L (Table 4.4 and Figure 4-13). Figure 4-12 suggests that the relationship between ozone Ct value and log inactivation of suspended HPC bacteria may be non-linear. However, it is difficult to characterize because most of the data at the higher Ct value (> 10 mg x min/L) were greater than the measurement limit. Comparison of ozone disinfection results for suspended HPC bacteria (Figure 4-12) and biofilm HPC bacteria (Figure 4-5) reveals that ozone was more effective against suspended HPC bacteria than

biofilm HPC bacteria. For biofilm HPC bacteria, inactivation was limited to 1 log-unit while for suspended HPC bacteria, inactivation was 3 to 4 log-units or greater than measurement limit, for the same ozone Ct value range. This may be due to (i) resistance to diffusion of ozone through the biofilm or (ii) reaction of ozone with components matrix of biofilm (i.e. EPS). These reactive-diffusion limitations may have resulted in a much lower ozone concentration at the biofilm bacterial cell walls or membranes than in the bulk solution (Allison et al., 2000; Stoodley-Hall et al., 2004).

### 4.3.2 Free Chlorine Disinfection of Suspended HPC Bacteria

Table 4-5 summarizes the results of all free chlorine disinfection experiments for suspended HPC bacteria at pH 6.9 and pH 9.0. Figure 4-13 and Figure 4-14 represent the log inactivation of suspended HPC bacteria by free chlorine in pH 6.9 and pH 9.0 phosphate buffers.

**Table 4-5:** Results summary of free chlorine disinfection experiments of suspended HPC bacteria using pipe wash water sample diluted (1:10) in 0.05 M buffer at 22°C

| Trial no. | pH | contact time, t | applied chlorine dose | avg. chlorine residual | free chlorine Ct | initial HPC | final HPC | log inactivation |
|---|---|---|---|---|---|---|---|---|
| | | min | mg/L | mg/L | mg x min/L | CFU/100 mL | CFU/100 mL | log-units |
| 1 | 6.9 | 1440 | 58.40 | 32.01 | 46,094 | 520,000 | 0 | >-5.72 |
| 2 | 6.9 | 60 | 42.95 | 32.49 | 1,949 | 520,000 | 0 | >-5.72 |
| 3 | 6.9 | 480 | 41.80 | 30.95 | 14,856 | 330,000 | 0 | >-5.52 |
| 4 | 6.9 | 240 | 32.44 | 24.25 | 5,820 | 330,000 | 0 | >-5.52 |
| 5 | 6.9 | 720 | 44.80 | 30.72 | 22,118 | 28,000 | 0 | >-4.45 |
| 6 | 6.9 | 1440 | 52.21 | 31.15 | 44,856 | 24,000 | 0 | >-4.38 |
| 7 | 6.9 | 480 | 39.06 | 32.89 | 15,787 | 27,000 | 0 | >-4.43 |
| 8 | 6.9 | 240 | 39.55 | 31.13 | 7,471 | 26,000 | 0 | >-4.41 |
| 9 | 6.9 | 720 | 57.29 | 29.11 | 20,959 | 420,000 | 0 | >-5.62 |
| 10 | 6.9 | 60 | 34.83 | 30.65 | 1,839 | 23,000 | 0 | >-4.36 |
| 11 | 9.0 | 5 | 30.05 | 28.14 | 141 | 14,100 | 20 | -2.85 |
| 12 | 9.0 | 5 | 30.05 | 28.66 | 143 | 15,100 | 19 | -2.90 |
| 13 | 9.0 | 1440 | 40.12 | 30.41 | 43,790 | 11,300 | 0 | >-4.05 |
| 14 | 9.0 | 60 | 35.21 | 32.06 | 1,924 | 14,000 | 0 | >-4.15 |
| 15 | 9.0 | 1440 | 40.12 | 33.00 | 47,520 | 14,600 | 0 | >-4.16 |
| 16 | 9.0 | 60 | 35.21 | 30.51 | 1,831 | 11,100 | 0 | >-4.05 |

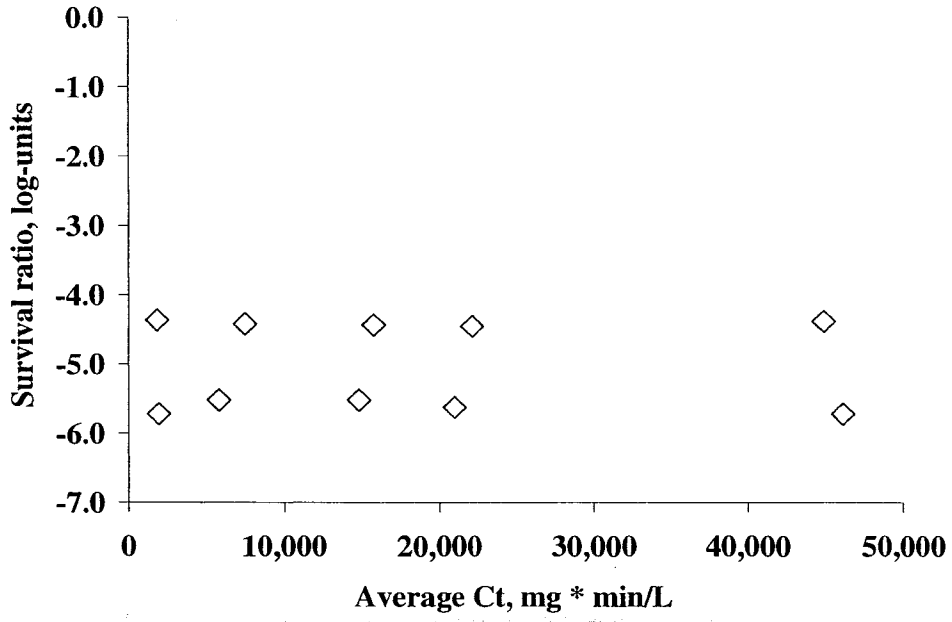


**Figure 4-13:** Survival of suspended HPC bacteria versus free chlorine Ct value at 22°C, at pH 6.9 (all results greater than measurement limit)

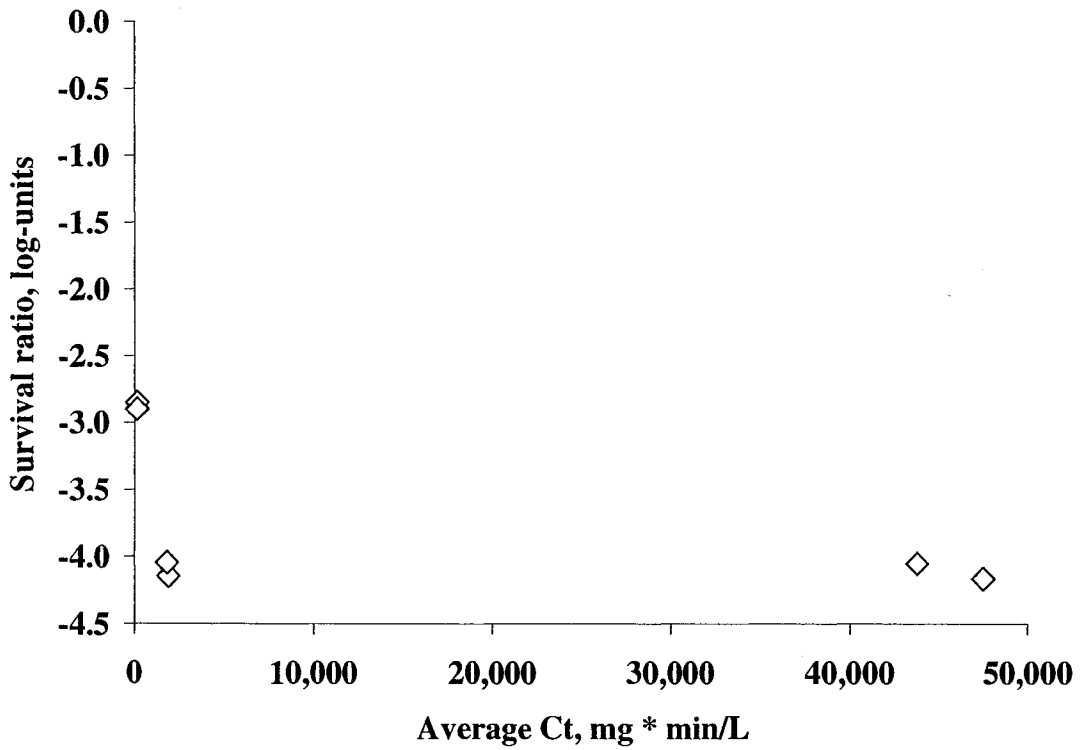


**Figure 4-14:** Survival of suspended HPC bacteria versus free chlorine Ct values at 22°C, at pH 9.0

Figure 4-13 and Figure 4-14 show that mostly complete log inactivation of suspended HPC bacteria was achieved at free chlorine Ct range of 1,839 to 46,094 mg × min/L at pH 6.9 (Runs 1 to 10) and at free chlorine Ct range of 141 to 47,520 mg × min/L at pH 9.0 (Runs 11 to 16), respectively. Comparing these results with free chlorine disinfection results for biofilm HPC bacteria (Figure 4-9), it was found that free chlorine was effective against both biofilm and suspended HPC bacteria. However, free chlorine was relatively more effective against suspended HPC bacteria than biofilm HPC bacteria. For example, for suspended HPC bacteria, at Ct value of < 2,000 mg × min/L, inactivation was greater than 4 log-units (Figure 4-13). While for biofilm HPC bacteria, inactivation was limited to ~ 2 log-units at Ct value of < 2,000 mg × min/L (Figure 4-9).

Figure 4-13 shows the suspended HPC bacteria inactivation of greater than measurement limit at all Ct values, from 1,839 to 46,094 mg × min/L (Runs 1 to 10 in Table 4-5). However, Figure 4-11 shows the increase in log inactivation of biofilm HPC bacteria with increase in free chlorine Ct value, at < 10,000 mg × min/L. The free chlorine disinfection results for biofilm HPC bacteria (Figure 4-9) and for suspended HPC bacteria (Figure 4-13) indicate that there was only 1.55 log inactivation of biofilm HPC bacteria at Ct of 1,710 mg × min/L (Run 1 in Table 4-2) in comparison to over 4 log inactivation (or greater than measurement limit) of suspended HPC bacteria at Ct of 1,839 mg × min/L (Run 10 in Table 4-5). It is already discussed that ozone was more effective against suspended HPC bacteria (Figure 4-12) than biofilm HPC bacteria (Figure 4-5). For biofilm HPC bacteria, inactivation was limited to 1 log-unit while for suspended HPC bacteria, inactivation was greater than 3 to 4 log-units, for the same ozone Ct value range. This indicates that, when the bacteria are attached to surfaces (biofilms), they are more resistant to ozone and free chlorine disinfectants. However, the difference between biofilm and suspended HPC bacteria log inactivation was much greater in the case of ozone. The potential reasons may be (i) the application of low ozone Ct value in comparison to free chlorine Ct value or (ii) ozone is far less stable and reactive chemical than free chlorine. Ozone has greater redox potential (2.07 volts) than free chlorine (1.36 volts) i.e. ozone is stronger oxidizing agent than free chlorine (Sawyer et al., 2003).

## 4.5 COMPARISON OF EXPERIMENTAL RESULTS WITH AVAILABLE LITERATURE AND THEIR SIGNIFICANCE FOR FIELD APPLICATIONS

deBeer et al. (1994) measured chlorine concentration profiles in 1-week old biofilms (150- to 200-$\mu$m thick) using ~10-$\mu$m chlorine microelectrode. Their biofilms were comprised of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae,* and were grown on stainless steel slides. The results of their chlorine profiles showed the gradual decrease of chlorine concentration from the biofilm boundary layer to the biofilm substratum surface when compared with the chlorine concentration in the bulk solution. It was determined that the chlorine concentration at the biofilm surface was only 20 to 30% of the chlorine concentration in the bulk solution. They attributed this phenomenon (decreased chlorine concentration into biofilm) to a reaction-diffusion interaction of chlorine with biofilms. They also showed that the chlorine penetration into biofilms is not a function of biofilm thickness. The nature of growth of biofilms and their morphology may result in producing higher EPS density or more resistant species (persisters) within biofilm, resulting in less efficacy of chlorine inactivation of biofilms.

A similar study was conducted by Chen and Stewart (1996) who determined the penetration of chlorine into laboratory grown biofilms of *Pseudomonas aeruginosa.* Their biofilms were grown on a stainless steel slide and the cells were dispersed in agarose gel slabs. They found that rate of penetration of chlorine into these experimental agarose biofilms was much slower when compared to the rate of penetration of chlorine into control biofilms containing no agarose. The chlorine was detected at the substratum of 773-$\mu$m thick control biofilms within 10 minutes. In comparison, only 10% of bulk solution chlorine was detected at the substratum of a 526-$\mu$m thick experimental agarose biofilm even after 3-hours contact time. They concluded that penetration of chlorine into biofilms was limited by the presence of exopolymeric substances and a reaction-diffusion mechanism.

Huang et al. (1995) conducted a study to determine respiratory gradients within bacterial biofilms of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (grown on stainless steel coupons), using monochloramine as a disinfectant. Treating biofilms with a monochloramine dose of 2 mg/L for 2 hours, they found only an average 1.3 log inactivation of biofilms. Supplementing the results with epifluorescence micrographs of frozen biofilms, they found that the biofilm bacteria at the substratum had greater respiratory activity than the biofilm bacteria near the biofilm-bulk fluid interface. They also attributed this phenomenon to the reaction-diffusion interaction of monochloramine with biofilms.

In an earlier study, on determining concentration of disinfectant within biofilms, Stoodley et al. (1994) demonstrated that the concentration of disinfectants is relatively lower at the biofilm substratum in comparison to that at the biofilm-bulk liquid interface. Their biofilms were comprised of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae*.

In order to determine the effect of free chlorine residual on the biofilm HPC bacteria, LeChevallier et al. (1987) carried out experiments on field biofilms, taken from New Jersey potable water main system. The water pH and temperature were reported as 8.0 and 24°C, respectively. Their experimental results showed that the free chlorine was not very effective in inactivating biofilms. Using a free chlorine Ct value of 1 mg × min/L (1.0 mg/L for 1-h contact time), the biofilm inactivation was only 0.03 log-unit (initial number = $1.7 \times 10^2$ CFU/ml, final number = $1.6 \times 10^2$ CFU/ml). Investigating the results they found that the irregular pipe surface, and the production of EPS, may have contributed to the disinfection inefficiency of free chlorine against biofilm HPC bacteria. In a later study on biofilm *Klebsiella pneumoniae*, grown on glass surfaces, LeChevallier et al. (1988a) confirmed that the biofilm (*Klebsiella pneumoniae*) bacteria are more resistant to free chlorine than suspended bacteria. Comparing the free chlorine disinfection efficiency for biofilm bacteria with that of suspended bacteria, they showed that the relative chlorine efficiency can be up to 150 times lower. They also attributed the increased free chlorine disinfection resistance to biofilm age.

The attribution of EPS to disinfectant resistance is also reported by Heinzel (1998). In his study, he found that the biofilm EPS may resist the penetration of disinfectants to the cell membrane.

It was already reported (in Chapter 2) that ozone is very effective against suspended bacteria. For example, *Legionella pneumophila* levels were reduced by greater than 2 logs with a contact time of 5 minutes at ozone concentration of 0.21 mg/L (U.S. EPA, 1999). However, there is only one study on biofilm HPC bacteria disinfection, by Momba et al. (1998). They found 4 log inactivation of biofilm HPC bacteria by applying the ozone at 2.6 mg/L for a contact time of 5 minutes. This study did not provide the information about biofilm age or thickness. In a study, on inactivating dental unit water system (DUWS) biofilms (14 days old; geometric mean of $3.4 \times 10^4$ CFU/cm$^2$) by ozone, Walker et al. (2003) observed that ozone could only inactivate 65% of biofilm (viable) and this may be due to use of brass connectors in DUWS systems.

Comparing the above available literature (experimental studies) with this study, it is found that biofilms offer resistance to disinfectants (ozone and free chlorine). However, the disinfection resistance was more in the case of ozone. As there are not enough studies to compare the disinfection efficiency of ozone against biofilm bacteria therefore it is difficult to conclude that ozone was considerably less effective against biofilm HPC bacteria. Also, the ozone inactivation experiments were conducted at field conditions, and a limited data set is available. In the case of free chlorine, mostly greater than measurement limit inactivation was observed against both biofilm and suspended HPC bacteria. However, free chlorine was less effective against biofilm bacteria at Ct values of $< 2{,}000$ mg $\times$ min/L. This conclusion is in accordance with the above mentioned studies.

Although the experiments in this study were conducted at field conditions, care should be taken when applying the disinfection results to field applications. The biofilms grown in the laboratory may not be identical to the biofilm that grows on the water mains in the field. Also, the conclusions are based upon a limited dataset.

## 4.5 EXPERIMENTAL LIMITATIONS

There were a number of experimental limitations that resulted from carrying out the experiments under different conditions. The major limitation was to compare ozone and free chlorine under field application conditions, provided by Denver Water (project sponsor). Due to that, free chlorine Ct values were much greater than ozone Ct values.

Another practical limitation of this study was to establish cultivation period for biofilms of sufficient concentration in order to measure quantitative HPC bacteria inactivation in 3 to 4 log range. Later experiments were, therefore conducted with relatively thick biofilms. Latter experiments were conducted with thin biofilm samples once the biofilm growth dynamics were better established (Figure 4-3).

The third limitation was the confounding variables such as pH of phosphate buffer for biofilm disinfection experiments and age of biofilm.

# CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

## 5.1 CONCLUSIONS

Based upon the experimental investigation for the use of ozone as an alternative disinfectant for biofilm disinfection in new and repaired water mains, it is concluded that:

- For the conditions tested, the log inactivation of biofilm HPC bacteria by ozone was limited to 1 log-unit at the highest ozone Ct of 56.3 mg x min/L. In comparison, inactivation of suspended HPC bacteria was greater than 3 log-units (or greater than measurement limit) at ozone Ct of 62.6 mg × min/L. These results suggest that ozone was not effective against biofilms, under the conditions tested. This may be due to (i) the diffusion-limitation property of EPS of biofilm that restricted diffusion of ozone to cell wall and membrane of biofilm or (ii) reaction of ozone within biofilm that resulted in dilution of ozone concentration before it could reach all of the individual bacterial cells within the biofilm. The statistical analysis of ozone disinfection results shows that biofilm disinfection is not a function of ozone Ct value, for the conditions tested.

- The free chlorine was found mostly effective for both biofilm and suspended HPC bacteria as the inactivation was greater than measurement limit in most of biofilm and suspended HPC bacteria experiments. However, the results indicate that free chlorine was not as much effective against biofilms. For example, there was only 1.55 log inactivation of biofilm bacteria at Ct of 1,710 mg × min/L in comparison to over 4 log inactivation of suspended bacteria at Ct of 1,839 mg × min/L.

- For the conditions tested, the log inactivation of biofilm HPC bacteria by ozone was significantly less than the log inactivation of biofilm HPC bacteria by free chlorine. The biofilm HPC bacteria inactivation by ozone was up to 1 log-unit in comparison to over 4 log-units by free chlorine. It should be noted that, these disinfection results were obtained under field application conditions (with very different Ct values) as required by the project sponsor.

## 5.2 RECOMMENDATIONS

During this study, a number of experimental challenges were identified. Based upon these challenges, the following recommendations are made for future experimental work:

- The cultivated biofilms within a laboratory environment may not be representative of the biofilms on CML water mains under field conditions. Therefore, for application of laboratory results in the field, future experimental work should include measurement of biofilm concentration of field samples of CML water mains. These measurements could potentially be correlated to the laboratory growth curve (Figure 4-3) to determine appropriate growth periods for laboratory biofilms.

- The results of this study suggest that further experiments will need to be conducted to confirm the additional inactivation of biofilm bacteria i.e. more data at large Ct values. In this study, semi-batch reactor flasks were used for ozone disinfection experiments. Larger experimental Ct values can be evaluated by replacing the semi-batch reactor with a continuous-flow reactor apparatus.

- The significance of bacterial growth within surface pores and crevices of CML coupons, and the potential effect on disinfection should be investigated. Future investigations could include microscopic examination of coupon surface both before and after scraping. More aggressive scraping techniques such as high pressure water spray (as now used by Denver Water) should be investigated for its ability to scrape the biofilm bacteria.

- The homogenization technique that was used to disperse the biofilm bacteria, was not thoroughly evaluated. This might be a significant factor in interpreting the inactivation (disinfection) results. Therefore, it is suggested that in future studies a protocol should be developed for ensuring the enumeration of individual cells rather than micro clumps of bacteria.

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# APPENDIX "A"

# DETAILED MATERIALS AND METHODS

## A1. MATERIALS

### A1.1 Deionized Laboratory Water

Deionized (DI) laboratory water, used for preparing all the chemicals/reagents, was obtained from an Elga Maximum Ultra Pure Water System (Fisher Scientific Limited, Hampton, New Hampshire, USA) operated at a resistivity of at least 16 MΩ/cm.

### A1.2 Ozone-Demand Free (ODF) Water

ODF water was prepared by bubbling ozonized gas, produced from a Welsbach ozone generator (model: Welsbach T-816, The Welsbach Corporation, Phoenix, Arizona, USA), through 4L of water, stirred constantly on a stirring hotplate, for at least 40 minutes in a concentrated ozone solution (> 20 mg $O_3$/L). The flask was covered with aluminum foil and was left to stand overnight and was then boiled for at least 10 min. After cooling, the ODF water was transferred into storage bottles.

### A1.3 Oxidant-Demand Free Glassware

All the glassware used for ozone disinfection experiments (disinfection reactors, volumetric and Erlenmeyer flasks, sample vials, buffer solution bottles, stir bars, volumetric pipettes, and pipette tips) was cleaned in a dishwasher (model: 97-975, Fisher Scientific Limited, Hampton, New Hampshire, USA) using Sparkleen 2 detergent (chlorinated laboratory detergent, Fisher Scientific, USA) followed by rinsing first with acetic acid and then three times with DI water (Finch et al. 1987). The clean glassware was then made ODF by soaking overnight with ozone saturated water (~ 20 mg $O_3$/L) and was covered with aluminum foil. After pouring out the water the glassware was oven-dried at 75°C for overnight and was sterilized at 121°C (Castle Autoclave, Getinge-Castle Canada Ltd., Mississauga, Ontario, Canada) before use. ODF water and glassware are also chlorine demand-free therefore they could be considered oxidant-demand free.

### A1.4 Acid-Washed Glassware

All the glassware used for preparing reagents was soaked in concentrated $HNO_3$ solution overnight and then oven-dried.

## A2. CHEMICALS

### A2.1 Chemical Oxygen Demand (COD) Reagents

*a) Standard Potassium Dichromate Digestion Solution, 0.01667 M*

COD digestion reagent was prepared by dissolving 10.216 g of primary standard grade potassium dichromate ($K_2Cr_2O_7$), previously oven-dried at 103°C for 2 hours, 167 mL concentrated sulfuric acid ($H_2SO_4$), and 33.3 g mercuric sulfate ($HgSO_4$) in 500 mL DI laboratory water, diluted to 1000 mL DI water.

*b) Sulfuric Acid Reagent*

COD acid reagent was prepared by dissolving 9.715 g of reagent grade silver sulfate ($Ag_2SO_4$) crystals in 1000 mL concentrated $H_2SO_4$. The acid reagent was mixed regularly for 2 days before the use.

*c) Potassium Dihydrogen Phthalate (KHP) Standard*

KHP stock solution (1000 mg/L) was prepared by dissolving 850 mg KHP ($HOOCC_6H_4COOK$), previously oven-dried at 110°C for 2 h, in 500 mL DI diluting to 1000 mL. COD standards (50, 100, 200, 400, and 600 mg/L) were then prepared from the stock solution for the purpose of plotting a standard COD curve. The standards, prepared under sterile conditions, were refrigerated at 4°C and were always checked before use for the development of any visible biological growth.

### A2.2 Total Organic Carbon (TOC) Reagents

*a) Organic Carbon Stock Solution*

Organic carbon stock solution (2000 mg/L) was prepared by dissolving 425 mg oven-dried KHP in 50 mL DI water. The solution was preserved by reducing the pH to less than 2 by addition of 0.1 mL analytical grade concentrated $H_3PO_4$ and was diluted to 100 mL. KHP standards (10 and 400 mg/L) were then prepared from the stock solution for the purpose of appropriate injection based upon total carbon (TC) concentration of the sample. The standards, prepared under sterile conditions, were refrigerated at 4 °C and were always checked before use for the development of any visible biological growth.

*b) Inorganic Carbon Stock Solution*

Inorganic carbon stock solution (800 mg/L) was prepared by dissolving about 706 mg oven-dried $Na_2CO_3$ in 50 mL DI water and diluting the solution to 100 mL. Inorganic carbon standards (10 and 400 mg/L) were then similarly prepared and stored.

*c) Potassium Persulfate, $K_2S_2O_8$ 2% W/V Solution*

$K_2S_2O_8$ solution was prepared by dissolving 20 g $K_2S_2O_8$ in 500 mL DI water. The solution was preserved by reducing the pH to less than 2 by addition of 0.1 mL analytical grade concentrated $H_3PO_4$ and was diluted to 1000 mL. The solution was stored in amber bottles in the dark at room temperature and was prepared freshly each month.

**A2.3 Buffers, 0.05 *M***

Buffers (0.05 *M*) of different pH values (6.0, 6.9, and 9.0) were prepared for biofilm disinfection experiments. The solutions were made ODF, as described in section A1.2.

*a) pH 6.0 Phosphate Buffer*

Phosphate buffer of pH 6.0 was prepared by dissolving 6.396 g potassium dihydrogen orthophosphate ($KH_2PO_4$) and 0.426 g disodium hydrogen orthophosphate ($Na_2HPO_4$) in 1 L of DI water. The pH of phosphate buffer was checked with a pH meter (model: Accumet® BASIC AB 15, Fisher Scientific Ltd., Hampton, New Hampshire, USA) before and after making the solution ODF. The pH was adjusted by adding concentrated $H_3PO_4$ or 25*N* NaOH, as required.

*b) pH 6.9 Phosphate Buffer*

Phosphate buffer of pH 6.9 was prepared by dissolving 6.818 g $KH_2PO_4$ and 7.098 g $Na_2HPO_4$ in 1 L of DI water. The pH of phosphate buffer was checked and adjusted as before.

*c) pH 9.0 Borate Buffer*

pH 9.0 buffer was prepared by dissolving 3.091 g boric acid and 0.68 g NaOH in 1 L of DI water. The pH was checked and adjusted as before.

## Preparation of 0.05 *M*, pH 6.0 ODF Phosphate Buffer

$pH = pKa + \log([salt]/[acid])$

$6.0 = 7.2 + \log([salt]/[acid])$

$\log([salt]/[acid]) = -1.2$

$[salt]/[acid] = 10^{-1.2} = 0.063$

$[salt] = 0.063\ [acid]$

*[salt] + [acid] = 0.05 M*

$0.063\ [acid] + [acid] = 0.05\ M$

$1.063\ [acid] = 0.05\ M$

$[acid] = 0.047\ M$ ($KH_2PO_4 = 136$ g)

*[salt] + [acid] = 0.05 M*

$[salt] = 0.05\ M - [acid] = 0.05\ M - 0.047\ M = 0.003\ M$ ($K_2HPO_4 = 142$ g or $Na_2HPO_4$)

6.392 g/L $KH_2PO_4$

0.426 g/L $K_2HPO_4$

## Preparation of 0.05 *M*, pH 6.9 ODF Phosphate Buffer

$pH = pKa + \log([salt]/[acid])$

$6.9 = 7.2 + \log([salt]/[acid])$

$\log([salt]/[acid]) = -0.3$

$[salt]/[acid] = 10^{-0.3} = 0.501$

$[salt] = 0.501\ [acid]$

*[salt] + [acid] = 0.05 M*

$0.501\ [acid] + [acid] = 0.05\ M$

$1.501\ [acid] = 0.05\ M$

[acid] = 0.033 M ($KH_2PO_4$ = 136 g)

*[salt] + [acid] = 0.05 M*

[salt] = 0.05 M – [acid] = 0.05 M – 0.033 M = 0.017 M ($K_2HPO_4$ = 142 g or $Na_2HPO_4$)

4.488 g/L $KH_2PO_4$

2.414 g/L $K_2HPO_4$

**Preparation of 0.05 *M*, pH 9.0 ODF Phosphate Buffer**

pH = pKa + log ([salt]/[acid])

9.0 = 9.3 + log ([salt]/[acid])

log ([salt]/[acid]) = -0.3

[salt]/[acid] = $10^{-0.3}$ = 0.501

[salt] = 0.501 [acid]

*[salt] + [acid] = 0.05 M*

0.501 [acid] + [acid] = 0.05 M

1.501 [acid] = 0.05 M

[acid] = 0.033 M ($H_3BO_3$ = 10.81 g)

*[salt] + [acid] = 0.05 M*

[salt] = 0.05 M – [acid] = 0.05 M – 0.033 M = 0.017 M (NaOH = 40 g)

3.091 g/L $H_3BO_3$

0.68 g/L NaOH

### A2.4 Sodium Formate Solution, 1.0 *M*

To neutralize any residual ozone in the sample solution, 1.0 *M* sodium formate (H.COONa) solution (BDH Laboratory Supplies, Dorset, England) was prepared by dissolving 13.6 g of sodium formate in 100 mL DI water. The solution was then sterilized at 121°C and stored in the refrigerator at 4°C.

### A2.5 Standard Sodium Thiosulfate, 0.1 *N* $Na_2S_2O_3.5H_2O$

0.1 *N* $Na_2S_2O_3.5H_2O$ solution was prepared by dissolving 2.50 g of $Na_2S_2O_3.5H_2O$ crystals in 100 mL freshly boiled DI water. A few mL of chloroform ($CHCl_3$) were added to minimize bacterial decomposition. The solution was stored in the dark for about 2 weeks and was then sterilized at 121°C before use.

### A2.6 Ozone Measurement Reagents

#### a) *Indigo Stock Solution*

Indigo stock solution was prepared by dissolving 770 mg light-sensitive 5, 5′, 7- indigo trisulfonic acid (Sigma Chemicals, St. Louis, Montana, USA), potassium salt ($C_{16}H_7N_2O_{11}S_3K_3$), and 1 mL concentrated $H_3PO_4$ (Certified ACS o-phosphoric acid 85%) in 500 mL DI water.

The solution, being photosensitive, was covered with aluminum foil and kept in the dark at the room temperature and checked for the absorbance every 2 months. Fresh stock solution was prepared when the absorbance of a 100-fold dilution was found < 0.16 absorbance units/cm.

#### b) *Indigo Working Solution (Indigo Reagent)*

Indigo reagent was prepared by dissolving 50.00 mL indigo stock solution, about 5.8 g anhydrous sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$), and 3.5 mL concentrated $H_3PO_4$ in 500 mL DI water. The solution, covered with aluminum foil, was stored in the dark at the room temperature.

c) *Malonic Acid Reagent Solution*

To control any interference by manganese in the sample solution, a malonic acid ($HOOCCH_2COOH$) solution was prepared by dissolving 5 g reagent grade malonic acid in 100 mL DI water. The reagent was sterilized before use and stored in the refrigerator at 4°C.

## A2.7 Free Chlorine Measurement Reagents

*a) Potassium Permanganate Stock Solution*

$KMnO_4$ stock solution was prepared by dissolving 891 mg $KMnO_4$ in 500 mL DI water and diluting to 1000 mL. The solution was stored in the refrigerator at < 4°C.

b) *Potassium Permanganate Working Solution*

$KMnO_4$ working solution was prepared by adding 10.00 mL stock solution to 100 mL DI water. 1 mL of this solution, diluted to 100 mL DI water, produces a chlorine equivalent of 1.00 mg/L in the DPD (N, N-diethyl-p-phenylenediamine) reaction. The working solution was always prepared fresh.

c) *Chlorine Standards*

A series of $KMnO_4$ standards covering the chlorine equivalent range of 1 to 4 mg/L was prepared from $KMnO_4$ working solution to plot a standard chlorine curve. The standards were always prepared fresh on the day of measurement.

## A3. METHODS

### A3.1 Heterotrophic Plate Count (HPC) Bacteria

**a) Apparatus**

- homogenizer (model: PowerGen 700D, Fisher Scientific Ltd., Hampton, New Hampshire, USA)
- biological safety cabinet (model: 1284 Thermo Forma Class IIA/B3, LabTrader, Vista, California, USA)
- low-temperature incubator (model: 307A, Fisher Scientific Ltd., Hampton, New Hampshire, USA), operated at 25°C

- Stereoscopic microscope, operated at 10 × magnification (Nikon USA, Melvile, New York, USA)
- Vortex (model: Fisher Vortex Geniez®, Fisher Scientific Ltd., Hampton, New Hampshire, USA)
- filtration assembly with a vacuum pump
- membrane filter holders
- pre-sterilized membrane filters (Millipore, 0.45 μm, Black gridded, 47 mm sterile, Millipore Corporation, Billerica, Massachusetts, USA)
- Difco® Peptone (Fisher Scientific Ltd., Hampton, New Hampshire, USA)
- peptone dilution bottles, 200-mL capacity
- peptone delivery apparatus (model: Brewer automatic pipetting machine, SEPCO/Scientific Equipment Products Company, Baltimore, Maryland, USA), operated at a speed to deliver 30 mL
- R2A agar media (Difco™ R2A agar, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA)
- Fisher stirring hotplate, for preparing media
- autoclave (model: Castle® Autoclave, Getinge/Castle Canada Ltd., Ontario, Canada)
- Bunsen gas burner and forceps
- water bath, set at 50°C, for reducing the temperature of freshly sterilized R2A agar
- Fisher brand tight lid 47 mm pre-sterilized plastic Petri dishes
- reagent alcohol, 70% v/v (LabChem Inc., Pittsburgh, Pennsylvania, USA)
- ODF, sterilized Erlenmeyer flasks, 250- to 1000-mL capacity
- ODF, sterilized glass beakers, 250-mL capacity
- Fisher brand borosilicate glass disposable serological pipets, 10-mL capacity

**b) Procedure**

***i. Preparation of peptone and dilution bottles***

To improve the recovery of injured HPC bacteria at room temperature, 0.1% peptone was prepared volumetrically by dissolving 2 g peptone in 2 L DI water. The ninety-mL of peptone dilution water was dispensed into dilution bottles using a Brewer automatic

pipetting machine and then the bottles were sterilized at 121°C for 15 minutes. They were used after cooling to room temperature.

### *ii. Preparation of R2A agar media*

To prepare 250 mL of media, 4.55 g of R2A agar in 250 mL of DI water (500 mL Erlenmeyer flask) was heated to dissolution during constant stirring on a stirring hot plate. The media was then transferred to 250 mL Erlenmeyer flasks. The flasks were covered with aluminum foil, and sterilized at 121°C for 15 minutes. The media was then cooled to 50°C in a water bath for about 30 minutes before pouring into Petri dishes.

### *iii. Preparation of Petri dishes*

Pre-sterilized Petri dishes were prepared by dispensing about 5-mL portions of R2A agar media inside a horizontal-flow laminar hood. These dishes were stored at <4°C and were used within two weeks of preparation.

### *iv. Preparation of biofilm samples*

To ensure monodispersion of HPC bacteria scraped from the coupons and to reduce the impact of clumping, the contents of the beaker (scraped biofilm in buffer solution) were homogenized for 3 minutes using a laboratory tissue homogenizer set at operating speed of 25,000 rpm (model: PowerGen 700D, Fisher Scientific Ltd., USA).

The HPC concentration was then determined using membrane filtration method (described later in this section). This measurement provided an estimate of the initial HPC concentration on the biofilm coupon, prior to disinfection experiments.

### *v. Sample preparation*

For counting the HPC bacteria in the range of 20 to 200 colonies per plate (according to Standard Methods, APHA, 1999), treated (disinfected) and untreated (non-disinfected or control) samples were diluted. The sample dilutions were prepared by sequentially pipetting 10 mL of sample into 90 mL peptone solution. Each diluted sample was well-mixed before filtering by about 10 complete back-and-forth movements.

### *vi. Bacterial Enumeration*

Each sample dilution was filtered through a pre-sterilized membrane filter (Millipore, 0.45 μm, Black gridded, 47 mm sterile, Millipore Corporation, Billerica, Massachusetts, USA), under partial vacuum. The filter was then placed on a petridish containing R2A agar. The Petri dishes were inverted and incubated at 25°C for 5 days.

The colonies on nine squares, selected at random (seen through microscope at one time), on each plate were counted with the aid of 10 × magnification stereo-microscope. The Petri dishes were tilted at about 45° angle on microscope stage to assist in counting. The results are reported as colony-forming units (CFU/mL) by rounding off the average to two significant figures.

### *vii. Calculation*

The bacterial log inactivation ratio was determined as under:

$$- \log S = - \log (N/N_0)$$

where,

| | | |
|---|---|---|
| S | = | inactivation ratio (no units) |
| log S | = | log inactivation ratio (no units) |
| N | = | concentration of bacteria in original suspension (CFU/mL) or on coupon surface (CFU/cm$^2$) after disinfection treatment |
| $N_0$ | = | concentration of bacteria in original suspension (CFU/mL) or on coupon surface (CFU/cm$^2$) before disinfection treatment |

## Appendix "B"

## Biofilm Reactor – Setup and Operation

## B1. Annular Biofilm Reactor Configuration

The Annular biofilm reactor consisted of two concentric glass cylinders; a stationary outer cylinder (water jacket for temperature control), and an inner cylinder housing a polycarbonate rotating drum to accommodate twenty removable CML coupons (see Appendix "C" for all the physical dimensions of the reactor, as measured).

The biofilm reactor (900 mL liquid capacity) was connected to a pipe wash water supply reservoir, consisting of a 10-L sterilized Nalgene® carboy. Three connections to the top of reservoir were provided for continuously circulating the water through the reactor with a pump and aerating the (inoculated) water via HEPA filter to maintain aerobic conditions for an aerobic biofilm growth.

The uniform mixing of water contents was achieved by a magnetic stirrer. The rotational speed of the drum ($\approx$ 25 to 430 rpm) was controlled via an electric motor on the top center of the reactor. Biofilm coupons were inserted and retrieved from the top port of the reactor (Figure 3.1).

## B2. Reactor Inoculation

The reactor was inoculated with pipe wash water for biofilm growth. The pipe wash water was freshly collected by Denver Board of Water Commissioners and samples of that water were shipped to University of Alberta by overnight courier every three weeks. An initial sample of this water was determined to be rich in HPC bacteria with a measured concentration of $66 \times 10^6$ HPC/100 mL. The water was dechlorinated using thiosulfite solution prior to use. Pipe wash water into the reservoir was partially replaced roughly every three weeks. The pipe wash water was not completely replaced to avoid any shock to biofilm bacteria.

## B3. Reactor Installation (Assembling the Parts)

The reactor was assembled according to Biosurface Technologies Corporation Operations Manual (Biosurface Technologies Corporation, Bozeman, Montana, USA). The installation steps are briefly outlined as follows:

- four stand-offs were fixed in stand-off holes on the aluminum base plate,
- the ball bearing was placed in the milled cup on the base plate ball tower,
- the rotating drum was placed on the ball bearing,
- the inner and outer cylinders were aligned with O-rings,
- the combined top aluminum plate was placed and aligned with stand offs and threaded steel rods,
- brass knurled nuts were tightened, and
- the motor/drive unit was mounted on the bearing house top plate

## B4. Flow Rate Determination

Using a 1 to 100 rpm pump (model: 7553-80, Cole-Parmer Instrument Company, Vernon Hills, Illinois, USA) with a compatible pump head (model: 77200-60 Easy Load II, Master flex® L/S™) the Annular reactor was run at a flow rate of 50 mL/min.

## B5. Rotational Speed of Biofilm Reactor

The rotational speed of the reactor drum was determined by filling the reactor with DI water and counting the revolutions (observing the white nylon spot on the top of the reactor seen through the top plate). The biofilm reactor was operated with a drum rotor speed of 10 rpm, to provide gentle mixing and a low shear environment.

## B6. Collection of Pipe Wash Water Samples

The pipe wash water samples were collected from the bottom port of the reservoir that otherwise remained covered with aluminum foil. To collect the samples the water was allowed to run slowly for about a minute to flush the sample port water and the desired volumes of samples were collected.

The samples were either analyzed on the same day for COD and TOC or stored at a temperature of 4°C for maximum of one week.

## B7. Preparation and Installation of Coupons

### *i. Preparation of Coupons*

The biofilm coupons (15 cm long x 0.5 cm wide) with a 0.5 mm thick layer of cement mortar supported on a plastic material were used for biofilm experiments. The purpose of using CML coupons was to interpret disinfection results with reference to new and repaired CML water mains disinfection. These coupons were provided by Biosurface Technologies Corporation (Bozeman, Montana, USA).

After removing coupons from the manufacturer's package, they were dipped into a detergent solution (RBS® 35 detergent concentrate, MJS Biolynx Inc., Canada), for overnight to remove any grease or dirt. The solution was prepared by adding 10 mL detergent into 500 mL 50°C water. The coupons were then rinsed with plenty of DI water. To obtain standard surface coupon characteristics the pre-cleaned coupons were soaked in 2.0 *M* HCl solution for 2 h followed by rinsing thoroughly with sterilized DI water. The coupons were kept moist by immersing in sterilized DI water till their installation in the reactor.

### *ii. Installation of Coupons*

To identify individual coupons for biofilm experiments the inner drum locations above the coupon slots were marked with numbers from 1 to 20. For initial experiments, full size coupons were inserted into the beveled slots on the inner drum of the reactor. One-half of the coupon was used for determination of initial biofilm concentration ($CFU/cm^2$) and the other half was used in disinfection experiments, on random basis. In later stages of this study, some of the coupons were cut in half length-wise before inserting into the reactor, mainly to increase the number of biofilm samples for experiments.

## B8. Reactor Cleaning/Disinfecting

The reactor, with the coupons installed, was half-filled with DI water and the knurled nuts holding the top plate were loosened. The reactor and the influent line were wrapped with aluminum foil. The whole assembly was then placed into the autoclave for 15

minutes at a temperature of 121°C. After cooling, the knurled nuts were tightened, the motor drive was reassembled, and the coupons were inserted. To check for any lose connections prior to operation the entire system was flushed with 4 L of 70% v/v ethanol solution for 2 h followed by rinsing with 10 L of sterilized DI water.

### B9. Operational Problems (Troubleshooting)

During the 8-month continuous operation of the reactor for biofilm growth a number of problems were faced. After about 2 months, minor leakage occurred due to loosening of a tube connector at the top. Major leakage occurred after about 4 months as the external top of the reactor was found filled with water. After thorough investigation of all the tubing and connections it was found that the water leaked from the bottom of the motor assembly. The inside seal of the top plate was most probably damaged. It was not a major spill therefore the rotation of the drum was stopped during night time rather than shut the reactor down. After operating the reactor for an additional three months the leakage became worse as all the reservoir water leaked. The reactor was stopped and all the coupons were transferred into phosphate buffer and were used in disinfection experiments within a week.

## Appendix "C"

## Annular Reactor – Physical Dimensions

| | |
|---|---|
| SIZE: | 25.4 cm × 25.4 cm × 45 cm h |
| Double Base Plate | 26 cm |
| Inner plate - 1 | 19 cm |
| Inner plate - 2 | 15.5 cm |
| Inner plate - 3 | 7.5 cm (stainless steel) |
| Draft Tubes | @ # 3, 8, 13, and 18 coupon sides |
| Water jacket | Ht = 19 cm |
| | ID = 19.55 cm |
| | OD = 21.5 cm |
| | Wall thickness = 0.98 cm |
| Inner cylinder | Ht = 18 cm |
| | ID = 15.6 cm |
| | OD = 17 cm |
| | Wall thickness = 0.7 cm |

Difference b/w inner cylinder & water jacket = 1.8 – 1.9 cm

Ht. Gap b/w top plates = 0.2 cm

| | |
|---|---|
| Rotating Drum | Ht = 15.5 cm |
| | Width = 13.5 cm |
| Coupon Holder | Ht = 14.6 cm |

Draft tube opening = 1.2 cm (height = 15.5 – 0.7 = 14.8 cm)

Gap b/w rotating drum and inner cylinder = 0.8 cm

Ht. Diff. b/w rotating drum and inner cylinder = 1 cm

Central yellow rubber holding bearing ball, Inner side = 0.6 cm, Outer = 0.8 cm

Outer boundary = 1.9 cm

**Concrete Coupons – Dimensions**

| | |
|---|---|
| Length | 15 cm |
| Width | 5 cm |
| Concrete coating | 15 cm × 1 cm |
| Coating thickness | 0.5 mm |