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I, Woo Hyoung Lee, hereby submit this original work as part of the requirements for the degree of:

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Development and Use of Microelectrodes to Evaluate Nitrification within Chloraminated Drinking Water System Biofilms, and the Effects of Phosphate as a Corrosion Inhibitor on Nitrifying Biofilm

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Development and Use of Microelectrodes to Evaluate Nitrification within Chloraminated Drinking Water System Biofilms, and the Effects of Phosphate as a Corrosion Inhibitor on Nitrifying Biofilm

A dissertation submitted to the Division of Research and Advanced Studies of the University of Cincinnati in partial fulfillment of the requirements for the degree of Doctorate of Philosophy in the Department of Civil and Environmental Engineering of the College of Engineering 2009

by

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Abstract

The implementation of increasingly stringent regulations for trihalomethanes (THM) and haloacetic acids (HAA) in the United States has resulted in an increasing use of chloramine within the past two decades as a secondary disinfectant in the drinking water treatment industry. Along with the addition of chloramines comes the risk of nitrification in the distribution system due to the ammonia which is released during chloramine decay. Nitrification in drinking water distribution systems may result in degradation of water quality and subsequent non-compliance with existing regulations.

Meanwhile, orthophosphate (PO$_4^{3-}$) plays an important role in corrosion control by combining with lead and copper in plumbing materials; it is recommended to maintain a phosphate residual of at least 0.5 mg P/L and, if possible, a residual of 1 mg P/L is preferable. However, relatively little is known about the effect of phosphate on nitrifying biofilm in chloraminated drinking water distribution systems when it comes to addition of phosphate to the water distribution system.

The primary objective of this research was to develop, fabricate and evaluate microelectrodes to evaluate nitrification within chloraminated drinking water system biofilm, and to determine the effects of phosphate on nitrifying bacteria biofilm. Chlorine microelectrodes for measuring monochloramine and phosphate microelectrodes for detecting phosphate ions in the biological sample (i.e. biofilms, aggregates) were developed, characterized and applied for in-situ environmental analyses. Both microelectrodes showed excellent selectivity toward target constituents and were successfully applied.
Monochloramine penetrated fully into nitrifying biofilms within 24 hours when fed at a 4:1 Cl\textsubscript{2}:N ratio, showing a cessation of aerobic activity via DO penetration following application of monochloramine. However, monochloramine penetration did not necessarily equate to a loss in viability, and the presence of excess ammonia in the water system prevented microbial inactivation. Biofilm recovery occurred when disinfection stopped. Monochloramine showed greater penetration compared to chlorine. Monochloramine penetrated into the biofilm surface layer 49 times faster than chlorine within the nitrifying biofilm and 39 times faster in the multi-species biofilm than did chlorine. Phosphate was found to act positively on biofilm development and nitrification in the long term. Phosphate microprofiles showed that phosphate contents in the biofilm was independent on the nitrifying activity. Low availability of phosphorus seemed to change biofilm structure at the biofilm surface. Phosphate did not affect the monochloramine penetration and monochloramine fully penetrated into the nitrifying biofilm within 24 hours both with and without phosphate.

The results of this research provide an improved insight into the relationship between phosphate as a corrosion inhibitor and nitrifying biofilm in chloraminated drinking water distribution systems, a better understanding of the impact of disinfectant (i.e. chlorine, monochloramine) penetration into biofilms on microbial activity changes (i.e. DO, ammonia, nitrate, and pH microprofiles), and understanding of the correlated viability achieved upon administration of chlorine or monochloramine disinfectant; this will allow development of better prevention and control strategies for nitrification episodes in the presence of phosphate, including for biofilm control.
Acknowledgement

“Blessed is the man who trusts in the Lord, whose confidence is in him. He will be like a tree planted by the water that sends out its roots by the stream. It does not fear when heat comes; Its leaves are always green. It has no worries in a year of drought and never fails to bear fruit.” – Jeremiah 17:7-8

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Chapter 1. Introduction

1.1. Introduction

Chloramination has replaced free chlorine for secondary disinfection at many utilities due to the trihalomethanes (THM) and haloacetic acids (HAA) regulations. However, utilities must consider the potential for nitrification in their monitoring and maintenance practices when using chloramines. Nitrifying bacteria, which are responsible for nitrification in the distribution system, are well known to grow in clumps or aggregates by adhering to a solid surface (i.e. plumbing materials). The presence of biofilm in drinking water systems is undesirable and may result in degradation of water quality and subsequent non-compliance with existing regulations and, therefore, effective biofilm control may be necessary to maintain the safety of public health. Many biofilm control strategies have been studied, and it was suggested that a less reactive biocide (i.e. monochloramine) that has a greater biofilm penetration than free chlorine be used, as it can result in better disinfection because of its persistence. However, the basis for biofilm resistance to chlorine and monochloramine biofilm penetration remains incompletely understood, and no direct measurements between monochloramine and chlorine have been conducted and compared with the penetration ability into the biofilm and its correlation to the microbial viability in the simulated drinking water distribution system biofilms.

Orthophosphate (PO$_4^{3-}$) plays an important role in corrosion control, combining with lead and copper in plumbing materials; it is recommended to maintain a phosphate residual of at least 0.5 mg P/L and, if possible, a residual of 1 mg P/L is preferable.
However, relatively little is known about the effect of phosphate on nitrifying biofilm in chloraminated drinking water distribution systems when it comes to addition of phosphate to the water distribution system.

The main objectives of this research are to develop and evaluate microelectrodes to measure monochloramine and phosphate within biofilms, and to apply these ammonia, dissolved oxygen (DO), nitrate and pH microelectrodes to the evaluation of nitrification within chloraminated drinking water system biofilms and the effect of phosphate as a corrosion inhibitor on nitrifying bacteria biofilms. A chlorine microelectrode using platinum wire was fabricated and evaluated for use in \textit{in-situ} measurement of monochloramine inside nitrifying biofilm under various disinfection conditions. Cobalt-based microelectrode was developed to measure phosphate in biofilms and used for determination of phosphate’s effective diffusion coefficient. Drinking water distribution systems were simulated using annular biofilm reactors, under controlled laboratory conditions, to provide biofilm samples for disinfection experiments and microprofiling, and to determine the effect of phosphate on nitrifying biofilm development and nitrification in a simulated water distribution system with various phosphate concentrations. Monochloramine penetration and its correlated effects on nitrifying biofilm activity and viability in chloraminated water systems were investigated and evaluated using microelectrodes and confocal laser scanning microscopy (CLSM) with \textsc{LIVE/DEAD}® \textsc{BacLight}™. Disinfectant penetrations of chlorine and monochloramine were also compared, and the impact of disinfectant penetration on viability of biofilms and the diffusion-reaction interaction between monochloramine and chlorine were investigated and evaluated for biofilm control. The effect of phosphate on nitrifying
biofilm development and nitrification in a simulated water distribution system with various phosphate concentrations was determined. The effect of various phosphate concentrations on changes in nitrifying biofilm structure was also evaluated, and the monochloramine penetration was compared with and without the presence of an orthophosphate residual using microelectrodes and CLSM.

1.2. Research Objectives

The main objectives of this research are to develop and evaluate microelectrodes to measure monochloramine and phosphate within biofilms, and to apply these ammonia, dissolved oxygen (DO), nitrate and pH microelectrodes to evaluate the nitrification within chloraminated drinking water system biofilms and the effect of phosphate as a corrosion inhibitor on nitrifying bacteria biofilms.

Specific research objectives are:

Object 1. Fabrication and Evaluation of Phosphate Microelectrode for In-Situ Environmental Analysis

- Development and characterization of phosphate microelectrodes for in-situ microprofile measurements in biological samples (e.g. biofilms, activated sludge floc)
- Evaluation of the performance of phosphate microelectrodes used in various environmental conditions and their application for environmental analysis (i.e. determination of a phosphate diffusion coefficient in enhanced biological phosphate removal (EBPR) process)
Object 2. Fabrication and Evaluation of Chlorine Microelectrode for Measuring Monochloramine within a Drinking Water Distribution System Biofilms

- Development and characterization of monochloramine microelectrodes for in-situ microprofile measurements in biofilms
- Evaluation of the performance of chlorine microelectrodes used in various monochloramine disinfection conditions

Object 3. Investigation into Monochloramine Biofilm Penetration: Penetration and its Effect on Biofilm Activity and Viability

- Direct measurement of monochloramine penetration into nitrifying biofilm with time during monochloramine disinfection
- Comparison of monochloramine penetration with and without excess ammonia during disinfection
- Evaluation of monochloramine disinfection with biofilm penetration and microbial activity changes (i.e. DO, ammonia, nitrate, and pH microprofiles), and viability upon the administration of chloramine disinfectant
- CLSM analyses with fluorescent probes for progression of viability during the disinfection

Object 4. Comparison of Disinfectant Penetration and Reaction-Diffusion Interaction between Monochloramine and Chlorine in Water Distribution Systems Biofilms

- Direct comparison of disinfectant penetration into various biofilms (i.e. nitrifying biofilms and multi-species biofilms) between monochloramine and
chlorine under various disinfection conditions (e.g. contact time, concentration, pre-chlorination, booster chlorination, and pre-chloramination)

- Investigation and evaluation of the diffusion-reaction interaction between monochloramine and chlorine for biofilm control
- Evaluation of disinfectant penetration and its correlated microbial activity changes (i.e. DO microprofiles) and viability upon the administration of chlorine and monochloramine disinfectant
- CLSM analyses with fluorescent probes for progression of viability during the disinfection

Object 5. The Effects of Phosphate as a Corrosion Inhibitor on Nitrifying Biofilms and Monochloramine Penetration in a Drinking Water Distribution System

- Inoculation and nitrifying biofilm development using annular biofilm reactors
- Continuous monitoring of nitrification during reactors operation
- Determination of the effect of phosphate on nitrifying biofilm development and nitrification in a simulated waster distribution system with various phosphate concentrations (i.e. 0, 1.0, and 12 mg P/L)
- Evaluation of the effect of various phosphate concentrations on nitrifying biofilm structure changes under CLSM with fluorescence probes
- Comparison of monochloramine penetration and microbial activity changes with and without the presence of an orthophosphate residual using microprofiles
- Determination of the *in-situ* biofilm profiles for ammonia, DO, nitrate, pH and phosphorus
- Evaluation of the effects and role of corrosion inhibitors (i.e. orthophosphate) on nitrifying bacteria biofilms in the chloraminated water distribution system

### 1.3. Research Hypothesis

The hypotheses of this research are:

1. Nitrification in chloraminated drinking water distribution systems is primarily due to biofilm.
2. Addition of phosphate can act positively on the biofilm formation including biofilm thickness and microbial activity (e.g. nitrification in this research) in the chloraminated drinking water.
3. Monochloramine penetrates more quickly and further into the biofilm than chlorine.
4. Monochloramine and free chlorine act differently at surfaces of biofilm composed of various bacteria

Therefore, biofilms which have the appropriate thickness for micro-profiling were developed and evaluated for study of the effects of phosphate on nitrification.

### 1.4. Research Significance

The primary objective of this research was to develop, fabricate and evaluate microelectrodes to evaluate nitrification within chloraminated drinking water system
biofilms, and to determine the effects of phosphate as a corrosion inhibitor on nitrifying bacteria biofilm.

Chlorine microelectrodes for measuring monochloramine and!phosphate microelectrodes for detecting phosphate ion in biological samples (i.e. biofilms, aggregates) were developed, fully characterized and applied for in-situ environmental analyses. Both microelectrodes showed excellent selectivity toward target constituents and successful biological application. Monochloramine penetration and chlorine penetration in the nitrifying biofilm were measured and compared with time combined with DO microprofiles and the viability test using LIVE/DEAD® BacLight™. Many researchers have used only viable counts and no direct measurement has been conducted to prove the in-situ disinfectant penetration in biofilm with time. This research successfully proved the hypotheses that monochloramine penetrates more quickly and further into the biofilm than chlorine and monochloramine and free chlorine act differently at surfaces of biofilm composed of various bacteria. This research also provides an improved insight into the relationship between phosphate and nitrifying biofilm in chloraminated drinking water distribution systems, and a better understanding of the impact of disinfectant (i.e. chlorine, monochloramine) penetration into biofilms on microbial activity changes (i.e. DO, ammonia, nitrate, and pH microprofiles) and their correlated viability upon the administration of chlorine or monochloramine disinfectant.

Overall, the results in this research can allow for development of better prevention and control strategies for nitrification episodes in the presence of phosphate, including biofilm control.
Chapter 2. Background

2.1. Chloramination Disinfection

2.1.1. Chloramine Chemistry

In aqueous solution, HOCl reacts with ammonia and forms inorganic chloramines according to the following equations (Snoeyink et al. 1980):

\[
\begin{align*}
\text{NH}_3 + \text{HOCl} & \rightarrow \text{NH}_2\text{Cl} + \text{H}_2\text{O} \quad \text{............ Monochloramine (pH>8.5)} \\
\text{NH}_2\text{Cl} + \text{HOCl} & \rightarrow \text{NHCl}_2 + \text{H}_2\text{O} \quad \text{............ Dichloramine} \\
\text{NHCl}_2 + \text{HOCl} & \rightarrow \text{NCl}_3 + \text{H}_2\text{O} \quad \text{............ Trichloramine}
\end{align*}
\]

Monochloramine and dichloramine coexist at a pH value of 4.5 - 8.5, and trichloramine occurs at pH <4.5. Monochloramine is the predominant chloramine formed at the pH range usually encountered in water treatment plants (pH = 6 - 9). In the presence of ammonia, the chlorine residual reaches a peak (formation of mostly monochloramine: chlorine to ammonia-N ratios = 4:1 - 5:1) and then decrease to a minimum called the breakpoint. The breakpoint, where the chloramine is oxidized to nitrogen gas, occurs when the chlorine to ammonia-N ratio is between 7.5:1 and 11:1.

\[
2\text{NH}_3 + \text{HOCl} \rightarrow \text{N}_2 + 3\text{H}_2\text{O} + 3\text{HCl}
\]

Addition of chlorine beyond breakpoint chlorination ensures the existence of a free available residual.
2.1.2. Advantages and Disadvantages of Chloramination

The Denver Water Department has been successfully using chloramination for more than 70 years for water treatment (Dice 1985), and many plants with high coliform counts in their distribution systems have switched from free chlorine to chloramines. Approximately 30 percent of U.S. drinking water plants use chloramines for disinfection (AWWA 2000: Betts 2002). Chloramine offers several benefits, including lower DBPs such as trihalomethanes and haloacetic acids, lower coliform regrowth in distribution pipes, and improved maintenance of disinfectant residual (Norton et al. 1997). LeChevallier et al. (1990) suggested the use of free chlorine as a primary disinfectant in water distribution systems and to convert the residual to monochloramine, if biofilm control is the goal. Hospitals using free chlorine as the residual disinfectant are 10 times more likely to experience outbreaks of Legionnaires’ disease than those using monochloramine (Kool et al. 2000). Thus, it has been suggested to use free chlorine as a primary disinfectant in water distribution systems and to convert the residual to monochloramine if biofilm control is the goal (LeChevallier et al. 1990).

However, chloramination may promote the growth of nitrifying bacteria, which convert ammonia to nitrite and nitrate (Wilczak et al. 1996). Ammonia oxidizing bacteria (AOB) produce nitrite, which may exert a chloramine demand, thus contributing to the deterioration of water quality in drinking water distribution systems. Following are some disadvantages of chloramination: (1) it is a relatively weaker disinfectant that HOCl, and hence requires a much higher concentration (C) and longer contact time (t) for pathogen inactivation when used as a disinfectant; (2) it may cause nitrification problems in distribution systems; and (3) it can lead to the formation of some DBPs (e.g.,
nitrosamines, cyanogen chloride) which are not currently regulated (Nikolaou et al. 2007) but are thought to be more toxic.

2.2. Nitrification in the Chloraminated Drinking Water Distribution System

Nitrification is a potential problem for utilities that utilize chloramines as a disinfectant and may occur when excess ammonia, low chloramine residual, excessive detention times within the distribution system or temperature promotes the growth of nitrifying bacteria (Kirmeyer et al. 2004). Nitrification in the distribution system can have a number of adverse effects, including decrease in chloramines residual (Valentine 1984), increase in heterotrophic plate count (HPC), increase in NO₂⁻ and NO₃⁻ concentration and decrease in alkalinity, pH, and dissolved oxygen (DO) concentrations (Wilczak et al. 1996; Liu et al. 2005). While disinfectant loss does not directly impact public health, it can allow microbial growth to proceed and leave the distribution system more vulnerable to contamination events (Kirmeyer et al. 2004).

2.2.1. Biochemistry of nitrification

Nitrification is a microbial process in which ammonia is first oxidized to nitrite (eq 2.6), and then nitrite to nitrate (eq 2.7) that involves many groups of bacteria, the most common being *Nitrosomonas* and *Nitrobacter* (Grady et al. 1999).

\[
\text{NH}_4^+ + \frac{3}{2} \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+ \quad (2.6)
\]

\[
\text{NO}_2^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + 2\text{H}^+ \quad (2.7)
\]
Nitrosomonas and Nitrobacter are aerobic lithoautotrophic bacteria commonly found in the natural environment. Their growth rates are controlled by pH, light, temperature, oxygen concentration, substrate availability, and composition of the biological community (Holt et al. 1995; Watson et al. 1989). Therefore, nitrification is ubiquitous as long as ammonia is present.

2.2.2. Impacts of nitrification on drinking water system

The presence of nitrifying biofilm in drinking water systems including distribution systems, reservoirs, and treatment facilities can generate water quality issues (e.g. disinfectant depletion, coliforms occurrences) and compliance issues (e.g. violation of surface water treatment rules, total coliform rule, and lead and copper rule), and effective biofilm control may be necessary to maintain the public health safety. It is well known that nitrification can be generated due to the detrimental development of nitrifying biofilms that attach to pipe surfaces, which consist of ammonia oxidizing bacteria (AOB) and/or nitrite oxidizing bacteria (NOB), because of the resulting ammonia decay from chloramination in the drinking water (Wilczak et al. 1996; Kirmeyer et al. 2004; Wolfe et al. 1988; Wolfe et al. 1990). Nitrification within drinking water distribution systems reduces water quality, causes difficulties maintaining adequate disinfectant residual, and poses public health concerns, including exposure to nitrite, nitrate, and opportunistic pathogenic microorganisms (AWWA 2006).
2.2.3. Nitrification control and operational responses

The onset of nitrification can be detected by monitoring the parameters in Table 2.1 (AWWA 2006).

Table 2.1 Usefulness of water quality parameters for distribution system nitrification monitoring

<table>
<thead>
<tr>
<th>Parameter/ Usefulness</th>
<th>Very Useful</th>
<th>Useful</th>
<th>Limited Useful</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total chlorine</td>
<td>Nitrate-N 1)</td>
<td>Dissolved oxygen</td>
<td></td>
</tr>
<tr>
<td>Nitrite-N</td>
<td>Total ammonia-N</td>
<td>TOC</td>
<td></td>
</tr>
<tr>
<td>Free ammonia-N</td>
<td>HPC-R2A</td>
<td>Hardness</td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>pH</td>
<td>Alkalinity</td>
<td></td>
</tr>
<tr>
<td>Free chlorine 2)</td>
<td></td>
<td>AOB 3)</td>
<td></td>
</tr>
</tbody>
</table>

1) Very useful if background nitrite-N level is consistent.
2) Very useful during breakpoint chlorination (not routine monitoring).
3) Limited usefulness until rapid inexpensive enumeration methods are available.

Nitrifying bacteria can be partially controlled by (Kirmeyer et al. 2004):

1) Increasing Cl₂:NH₃-N ratios as high as 5:1 to eliminate excess ammonia

2) Maintaining stable chloramine residuals to minimize chloramine demand and decay and the release of ammonia

3) Performing breakpoint chlorination

4) Reducing distribution system residence times

5) Cleaning reservoirs and distribution system pipelines to remove biofilm buildup, sediments or tubercles

6) Keeping distribution system water temperatures low through blending or proper storage tank management to prevent thermal stratification
7) Flushing of distribution systems to remove the nitrified water and to introduce fresh water

8) Increasing the pH of the finished and distributed water

9) Increasing the chloramine residual in the distribution system

2.3. Biofilm

2.3.1. Biofilm in the drinking water systems

Biofilms are relatively thin layers (up to a few hundred micrometers thick) of microorganisms that form microbial aggregates and also attach and grow on surfaces (Bitton 2005). Observations of living biofilms by confocal laser scanning microscopy (CLSM) have helped in the understanding of biofilm structure. Biofilm takes days to months to develop, depending on nutrient availability and environmental conditions. The decreased diffusion of oxygen is conducive to the development of facultative and anaerobic microorganisms in the deeper layers of the biofilm. A number of processes contribute to biofilm development on surfaces exposed to water flow. The processes involved are the following: 1) surface conditioning, 2) transport of microorganisms to conditioned surfaces, 3) adhesion of microorganisms to surfaces, 4) cell anchoring to surfaces, 5) and cell growth and biofilm accumulation (Gomez-Suarez et al. 2002)
2.3.2. Microelectrode and microscopic techniques for biofilm analyses

Microelectrodes, with small tip sizes of 1 to 10 μm diameter, have been used widely in biofilm research. Constructed based on a variety of theories, microelectrodes can be utilized in studying the 3-dimensional micro-environmental profiles of important growth and activity parameters in a biofilm system, including dissolved oxygen, redox potential, pH value, essential ions like ammonia, nitrate, nitrite, sulfide, phosphate and potassium, and inhibitory ions like heavy metals (Bishop et al. 1999; Lu et al. 2002; Santegoeds et al. 1998; Schramm et al. 1996; Wang et al. 2005).

CLSM can be used to examine a biofilm in its natural hydrated state, avoiding the problems of fixation and dehydration, and can provide a computer generated three-dimensional depiction of the biofilm’s structure (Wimpenny et al. 1997). CLSM has been introduced for investigation of the 3-D structure and mass distribution in a biofilm system (Lawrence et al. 1999). CLSM has also been utilized to determine the diffusion coefficients in biofilms (Lawrence et al. 1994). The combination of CLSM and fluorescent agents can be used to demonstrate the distribution of heavy metals in biofilms. Utilizing multi-channel staining and a variety of probes for bacteria and even extracellular polymeric substances (EPS), the relationship among biofilm components can be demonstrated (Lawrence et al. 1998). However, the working penetration distances of the objectives used by CLSM are generally less than 200 μm, and thus CLSM is limited to the investigation of relatively thin and relatively young biofilms (Bishop 1997). CLSM focuses on a very small field in a biofilm and often can not demonstrate the whole micro-environmental profiles for thicker biofilms. As a result, in order to better describe
the 3-D structure and mass transport and distribution in a biofilm system, microelectrodes are essential and necessary (Peng 2007).

### 2.4. Phosphate as Corrosion Inhibitor in Drinking Water System

#### 2.4.1. Corrosion control

Lead and copper are being regulated by the National Primary Drinking Water Regulations for Lead and Copper (or the Lead and Copper Rule, LCR), which became effective in 1991, because of the possible negative health effects associated with drinking water containing these two contaminants. In this regulation, the action level for lead is 0.015 mg/L and the copper action level is 1.3 mg/L (USEPA 2003).

Orthophosphate (PO$_4^{3-}$) can play an important role in corrosion control, combining with lead and copper in plumbing materials as shown in Table 2.2. This orthophosphate as corrosion inhibitor reacts with dissolved metals including Ca, Mg, and Zn in the water to form a very thin microscopic metal-phosphate coating on a pipe surface that is exposed to the treated water in the water distribution system. As a result, lead and copper levels in the water will remain low. The key to ensuring that orthophosphate will reduce lead and copper levels is to maintain the proper pH and orthophosphate residual. Residual orthophosphate is the free amount of orthophosphate measured in the distribution system. It is very important for most water systems to maintain a residual of at least 0.5 mg/L as phosphate (P) and, if possible, a residual of 1 mg/L as P is preferable (USEPA 2003). When using orthophosphate for lead and copper control, the pH should be maintained within the range of 7.2 – 7.8. If the pH is too low, even high dosages of orthophosphate will not work. At high pH, poor corrosion-protecting film stability has often been
observed. Much higher concentrations are often needed to resolve copper problems than lead problems. Treatment chemicals, often containing zinc, will help protect cement and cement mortar-lined pipes. When copper or zinc concentrations in wastewater discharge or sludge are of concern, pH/DIC (Dissolved Inorganic Carbonate) adjustment to control copper corrosion is usually preferable, if feasible, for water quality maintenance.

Table 2.2 The effect of orthophosphate on corrosion control in drinking water*

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthophosphate</td>
<td>- Forms protective films on iron, galvanized pipe, and lead.</td>
</tr>
<tr>
<td></td>
<td>- Slows oxidation of copper at neutral pH.</td>
</tr>
<tr>
<td></td>
<td>- Tends to form colloidal lead and maybe other metal species at pHs above 8.</td>
</tr>
<tr>
<td></td>
<td>- Interferes with calcium carbonates nucleation and growth.</td>
</tr>
</tbody>
</table>


2.4.2. Impacts on biofilm growth

Zhang et al. (2008) proposed that nitrification in polyvinyl chloride (PVC) premise plumbing is insensitive to phosphate concentrations of 5 - 1,000 ppb. Some researchers indicate that phosphorus can affect microbial growth in drinking water (Miettinen et al. 1997; Keinänen et al. 2002, Lehtola et al. 2002), and nitrifying activity was immediately enhanced after addition of phosphate for the purpose of ammonia removal in one water treatment plant (Takayuki et al. 2002).
Chapter 3. Cobalt-based Phosphate Microelectrode for In-Situ Environmental Analysis

3.1. Abstract

The in situ monitoring of phosphate has been of great importance in many environmental applications, particularly those involving biological treatment processes and eutrophication monitoring. A microelectrode with small tip size (~10 μm) was fabricated with cobalt wire, characterized and evaluated for in situ and in vivo environmental analysis of phosphate in biological applications. The electrochemical performance of this cobalt-based microelectrode was fully examined for its characteristics, including detection limit, response time, selectivity, reproducibility, lifetime, interference with pH, ions and dissolved oxygen (DO), and the stirring effect. The microelectrode showed excellent selectivity for the orthophosphate ions \( \text{HPO}_4^{2-}, \text{H}_2\text{PO}_4^- \) in various environmental conditions. Alkalinity and DO were found to interfere with electrode response to phosphate. The phosphate microelectrode was also evaluated with Scanning Electron Microscopy (SEM) and Cyclic Voltammetry (CV). The developed microelectrode was used for in situ monitoring of phosphate in microbial flocs. Well-defined phosphate concentration microprofiles were measured within activated sludge flocs in the enhanced biological phosphate removal (EBPR) process, and a fluorescent in situ hybridization (FISH) and clone library analysis was also conducted to identify polyphosphate accumulating organisms (PAOs). The center of the flocs had the highest phosphate concentrations, and the stratification of the flocs found by microprofiling
indicated that the PAOs were probably distributed evenly throughout the flocs. Under the assumption that the phosphate, which was generated because of phosphate release by microbial activity, was not consumed by microbes and was only transferred from the flocs to the bulk by diffusion during anaerobic conditions, the effective diffusion coefficient \( (D_f) \) for phosphate release within the flocs was calculated to be \( 3.33 \times 10^{-7} \) \( \text{cm}^2/\text{s} \) at the end of the anaerobic phase of the EBPR process. This full characterization and successful application showed that the cobalt-based phosphate microelectrodes can be a very useful tool for \textit{in situ} measurement of phosphate in various environmental conditions, including within microbial flocs.

**Keywords:** Cobalt wire; diffusion coefficient; clone library analysis; enhanced biological phosphate removal (EBPR) process; in-situ monitoring; microelectrode; microbial flocs; phosphate; fluorescent in situ hybridization (FISH)

### 3.2. Introduction

The \textit{in situ} monitoring of phosphate has been of great importance to the fields of clinical, environmental, and horticultural analysis for over 3 decades (Meruva et al. 1996). For example, phosphate measurement has been needed to monitor the eutrophication process, control the amount of phosphorus supplied to crops in modern agriculture, and determine the phosphate concentration in body fluids for clinical diagnosis. Phosphate measurements also are needed to control the amount of phosphorus supplied to crops as fertilizer, and to determinate phosphate concentrations in body fluids for clinical diagnoses like hyperparathyroidism, vitamin D deficiency and Fanconi syndrome (Engblom 1998; Tissen 1995; Kawasaki et al. 1989). Hence, the need for inorganic
phosphate measuring methods with high accuracy for determining the true concentration is obvious. Burton (1973) indicated the problem with classical colorimetric methods, including the change of phosphate contents during acidification of the sample. The need for a simple, accurate, and compact device for monitoring phosphate was recognized, and efforts to develop phosphate sensors have been ongoing for three decades (Engblom 1998). Phosphate sensors, in the forms of a coated wire (Liu et al. 1989), liquid membranes (Glazier et al. 1988; Liu et al. 1997; Shkinev et al. 1985; Zarkinii et al. 1980), solid-state (Grabner et al. 1986; Petrucelli et al. 1996; van Staden 1993), metal (Chen et al. 1997; Meruva et al. 1996; Wang et al. 2005; Xiao et al. 1995) and biosensor (Male et al. 1991; Watanabe et al. 1988), have been described to accurately measure the phosphate concentration in environmental samples. However, the large sizes of the electrodes have caused difficulty in applying them to small volume samples, such as in biofilm and tissue research. Moreover, there are few applications published where phosphate sensors have been put to real use (Engblom 1998).

Many electroanalytical techniques have been developed to fabricate sensors, and significant effort has been given to the development of phosphate-selective electrodes (Glazier et al 1988; Watanabe et al. 1988; Liu et al 1990; Midgley et al. 1986; Carey 1997; Lui et al. 1997; Marco et al. 2003; Engblom 1999). In recent years, Xiao et al. (1995) introduced cobalt as a phosphate-sensitive electrode material and showed that oxidized surfaces of metallic cobalt could be used as a selective potentiometric sensor for phosphate (Xiao et al. 1995). They suggested a host-guest mechanism to explain the phosphate electrode response. Meruva et al. (1996) found that the phosphate electrode response depended on pH, ionic strength, buffer salts and stirring rate (Meruva et al.
They suggested a mixed potential response mechanism, in which a cobalt oxide layer should be first generated on the surface of the cobalt in deionized (DI) water or buffer solution without phosphate ion, and then used to detect the phosphate ion in the sample or standard solution. This cobalt oxide reacts with phosphate ions in the solution and forms cobalt phosphate precipitates at the electrode surface, depending on pH values in the solution (Eq. (3.1)-(3.3)). These reactions give a voltage output which can be converted to phosphate concentration (Xiao et al. 1995).

\[
\begin{align*}
3\text{CoO} + 2\text{H}_2\text{PO}_4^- + 2\text{H}^+ & \leftrightarrow \text{Co}_3(\text{PO}_4)_2 + 3\text{H}_2\text{O} \quad \text{at pH 4.0} \quad (3.1) \\
3\text{CoO} + 2\text{HPO}_4^{2-} + \text{H}_2\text{O} & \leftrightarrow \text{Co}_3(\text{PO}_4)_2 + 4\text{OH}^- \quad \text{at pH 8.0} \quad (3.2) \\
3\text{CoO} + 2\text{PO}_4^{3-} + 3\text{H}_2\text{O} & \leftrightarrow \text{Co}_3(\text{PO}_4)_2 + 6\text{OH}^- \quad \text{at pH 11.0} \quad (3.3)
\end{align*}
\]

A fabricated microelectrode was evaluated with static potentiometric techniques, in which the potential of an electrochemical cell is measured at equilibrium when no electrolysis is occurring and hence the net current is zero (Strobel et al. 1989).

The enhanced biological phosphorus removal (EBPR) process is operated using polyphosphate accumulating organisms (PAOs) that have the unique ability for luxury phosphate uptake and release during aerobic and anaerobic conditions, respectively, during the operation as shown in Figure 3.1. Therefore, in designing nutrient removal processes, many modeling efforts were made based on key factors of phosphate uptake and release rates which are governed by PAOs during the anaerobic and aerobic phases of the system operation (Satoshi et al. 2006; Erdal et al. 2003) and most EBPR processes have been operated with the cycle or continuous flow of these two conditions (i.e. anaerobic and aerobic condition). Many studies have been conducted to understand the mechanisms that are influencing the phosphorus removal capacity, including by
modifying operating conditions (Satoshi et al. 2006; Erdal et al. 2003). Several models have been introduced to elucidate the mechanisms of the phosphate removal process including the Activated Sludge Model (ASM) No. 2, which incorporates biological phosphorus removal with ASM 1 for dynamic simulation of combined biological processes for chemical oxygen demand (COD), nitrogen and phosphorus removal (Henze et al. 1995; Henze et al. 2000; Comeau et al. 1986; Gujer et al. 1995; Mino et al. 1997). However, these models have focused on macro-scale monitoring, including bulk solution analyses, operation mode, and modification of reactor type to enhance the phosphorus removal efficiency and to understand this mechanism. In addition, even though there are an ample number of phosphorus removal models available in the literature that are based on macro-scale monitoring, micro-scale research to evaluate the process analysis has been limited, as a result of the lack of necessary tools to measure the small floc samples. For better understanding of the phosphorus removal mechanism, there was a clear need for in situ monitoring of phosphate within the biofilms and/or activated sludge flocs, using microelectrodes with a very small diameter tip. Direct monitoring of phosphate in the flocs taken from the anaerobic portion of an EBPR oxidation ditch was reported using a phosphate ion-selective microelectrode (Wang and Bishop, 2005). Substrate concentration microprofiles reflect a mass balance which indicates the equilibrium between nutrient delivery and nutrient removal at each data point, and the shape of the concentration profile is affected by the biokinetic parameters of a metabolic microbial reaction (Lewandowski et al. 2003). These parameters can be derived from these profiles; it is important to evaluate these parameters because they are used in mathematical models for nutrient removal. In this chapter, microbial phosphate diffusion
kinetic analysis was introduced and the value was calculated here under successful PAO activity; and this value can be considered in modeling efforts.

Recently, microelectrodes and molecular analyses have been widely used in analyses of microbial communities and their kinetics in complex environmental systems (Gieseke et al. 2001; Okabe et al. 1999; Okabe et al. 2000; Schramm et al. 1996). Microelectrode measurements have been used to directly investigate and measure the microenvironment and activity of microorganisms in their habitats with a high spatial and temporal resolution and minimum disturbance, and have been known as the most reliable techniques. (de Beer et al. 1997; Okabe et al. 2000; Schramm et al. 1996). These microelectrodes provide local chemistry which indicates fine scale distributions of specific microbial populations gained from Fluorescent in situ hybridization (FISH) and their correlated microbial activity in the combined use of FISH techniques (Okabe et al. 2000).

Chapter 3 describes the performance of phosphate microelectrodes used in various environmental conditions and their application to actual environmental analysis (i.e. determination of a phosphate diffusion coefficient). The electrochemical performance of a cobalt-based microelectrode with very small tip size (~ 10 μm) was fully examined for its characteristics, including detection limit, response time, selectivity, reproducibility, life time, interference with pH, ions and dissolved oxygen, and the stirring effect. The response to phosphate was also evaluated with Scanning Electron Microscopy (SEM) and Cyclic Voltammetry (CV). The main microbial community in an EBPR process was indentified and their spatial distributions and microbial activity were investigated using microelectrode and molecular techniques. Phosphate concentration microprofiles were
measured within activated sludge flocs in an EBPR process using a cobalt-based phosphate microelectrode, which has a very small tip (5-10 μm), and the PAOs in activated sludge flocs that are responsible for a well-operated EBPR process were indentified using clone library analysis. The diffusion coefficient of phosphate ions in a water-floc matrix was also determined using microprofiles. These results provide a better understanding of the phosphate removal mechanism, and this understanding of the internal function of flocs can lead to improvement in the modeling, design, and operation of the biological phosphorus removal process.

3.3. Materials and Methods

3.3.1. Microelectrode fabrication

The phosphate microelectrode is a potentiometric sensor (Engblom 1998). In potentiometry, the voltage output (mV) is measured and converted to target constituent concentrations. Cobalt metal, which has a selective response towards dihydrogen phosphate, was used to detect phosphate in the sample solution (Xiao et al. 1995). The fabrication steps for the conventional needle-type phosphate microelectrode were based on the method of fabricating a solid-state ion-selective microelectrode (Yu et al. 1998). A section of cobalt wire (0.1 mm diameter, 99.995% pure, Aldrich Chemical Company), with an original diameter of 0.1 mm, was cut and etched in 2 M KCN until it reached 3 μm in diameter. After heating and pulling a glass micropipette (O.D.: 1.2 mm, I.D.: 0.69 mm, 15 cm length, Sutter Instrument Co.) over the flame of a small burner, the etched cobalt wire was inserted into the pulled glass micropipette; the middle section of this micropipette was then melted to seal the cobalt wire in the glass using heat from a trough.
heating filament (Sutter Instrument Co.). The tip, which was wrapped with glass, was then beveled on a diamond abrasive plate (Sutter Instrument Co.) with a 45° angle to expose the cobalt surface. Next, the microelectrode was assembled with copper wire using bismuth alloy (44.7% bismuth, 22.6% lead, 19.1% indium, 8.3% tin and 5.3% cadmium). The copper wire was then connected to a Bayonet Neill-Concelman (BNC) cable. The finished phosphate microelectrode has a 5-10 μm tip diameter which allows for measuring the phosphate concentration in biofilm or activated sludge flocs without destroying the biofilm structure (Fig. 3.2). Fig. 3.3 shows that the cobalt wire was well exposed and coated with a glass body, with a 45° angle taper.

3.3.2. Pretreatment

The phosphate microelectrode was connected with an Ag/AgCl reference milli-electrode (MI-401, Microelectrodes Inc.) and immersed into DI water to form a cobalt oxide (CoO) layer on the surface of the microelectrode tip. Fig. 3.4 shows schematically the experimental setup for characterization and evaluation of the phosphate microelectrodes. The potential between the two electrodes was then monitored using a millivolt (mV) meter (Model 215, Denver Instruments). After a stable potentiometric response was obtained below -250mV, the microelectrode was removed from the DI water and immersed in 10^{-4} M KH_{2}PO_{4} solution at pH 7.5 until a steady-state potential was observed. Through the pretreatment step, the cobalt-based phosphate ion microelectrodes achieved sensitivity towards the two main forms of phosphate ions (H_{2}PO_{4}^{-} and HPO_{4}^{2-}) of interest.
3.3.3. Calibration and evaluation of microelectrode performance

Based on the pH value at which typical EBPR systems are operated, the finished phosphate microelectrode performed linearly in the range 10^{-5} to 10^{-1} M of phosphate standard solution in the pH 7.5 buffer solution. Ortho-phosphate standard solution was prepared with KH$_2$PO$_4$, and the pH of each standard solution was adjusted by adding potassium hydroxide. A commercial Ag/AgCl milli-electrode (MI-401, Microelectrodes Inc.) was used as an external reference electrode. Evaluation of the finished phosphate microelectrodes was conducted at ambient oxygen levels and at room temperature (23°C) and all calibration measurements at each experiment were replicated three times. A commercial oxygen mini-electrode (OM-4 Oxygen sensor, Microelectrodes Inc.) was used to verify the oxygen concentration in the standard solution during the DO interference experiment. A pH meter (Model 15, Accumet) with a pH/ATC combination electrode (Cat. No. 13-620-112, Accumet) was used to verify the pH value during the experiment. A mV meter (Model 215, Denver Instruments) was used to obtain potentiometric responses, and a Balance Talk SLTM (Labtronics Inc.) spread logger was used to continuously collect data. All experiments were conducted in a Faraday cage to minimize electrical noise and at 23°C room temperature, as illustrated in Fig. 3.4. An Analyst 300 Furnace and Flame Atomic Absorption Spectrometer (FAAS) with a graphite furnace was used to measure aqueous cobalt metal concentrations. MINEQL+ (Environmental Research Software, Hallowell, ME) chemical equilibrium modeling system software was used to investigate the dominant species of phosphate under different pH conditions (Schecher et al. 2003).
3.3.4. Cyclic voltammetry

A BAS 100B electrochemical analyzer (Bioanalytical Systems Inc.) was used to record the cyclic voltammograms (CVs). An Ag/AgCl reference electrode was fitted with a Pt disk as the auxiliary electrode for voltammetry experiments. The scan rate was 50 mV/s when the total phosphate standard solution was varied from $10^{-5}$ to $10^{-2}$ M in 0.05 M KHP buffer solution at pH 4.0.

3.3.5. Surface characterization

Scanning electron microscopy (SEM) studies with energy dispersive X-ray spectroscopy (EDS) were carried out with a XL30ESEM-FEG (Philips XL Series) with a 25keV beam energy for characterization of the surfaces of cobalt microelectrodes before and after exposure to phosphate standard solutions.

3.3.6. Sequencing Batch Reactor (SBR) operation

A bench-scale sequencing batch reactor (SBR) with 4L volume was operated in the sequence of fill, mix, aerate, idle, settle, and decant phases for more than 6 months in an EBPR process mode to grow phosphate-accumulating organisms (PAOs), which are responsible for high removal efficiency in the phosphate removal system. The reactor was inoculated with mixed liquor from the return sludge stream at the Upper Mill Creek Water Reclamation Facility, West Chester, Ohio. Air was used for aeration during the aeration period, and mixing of the reactor was achieved using a magnetic stirrer. The system was equipped with a timer, Chrontrol® controller (Chrontrol corp. XT-4, San Diego, CA) and a solenoid valve to turn the following equipment on and off: air supply,
mixer, and the pump for feeding or supernatant decant. The influent feed was transferred to the reactor by a peristaltic pump (Cole-Parmer, Vernon, IL). The sludge retention time (SRT) was maintained at 10 days by wasting an appropriate amount of mixed liquor directly from the reactor at the end of the aerobic cycle. During the decanting period, two liters of supernatant were removed from the reactor, and the same amount of fresh feed was introduced during the subsequent fill period. The hydraulic retention time (HRT) was 8 hours, including 2 hours in the anaerobic phase (mix) and 3 hours in the aerobic phase (aeration). The concentration range of chemical oxygen demand was 200 to 250 mg/L in the influent and 5 to 10 mg/L in the effluent. The average influent phosphorus concentration and the effluent phosphorus concentration were approximately 2.6 to 3.3 mg/L and 0.2 mg/L, respectively. During the experiment periods, the mixed liquor suspended solids concentration (MLSS) was 1900 to 2000 mg/L. Synthetic wastewater of the following composition was used as the feed solution: 384.4 mg/L CH₃COONa (200 mg/L as COD basis), 33.7 mg/L KH₂PO₄ (7.5 mg/L as PO₄³⁻-P), 93.3 mg/L (NH₄)₂SO₄ (20 mg/L as NH₄⁻-N), 10.5 mg/L CaCl₂, 90 mg/L MgSO₄·7H₂O, 1 mg/L yeast extract and 0.3 mL of nutrient solution per liter. Phosphate (PO₄³⁻-P) and nitrate (NO₃⁻-N) concentrations in the bulk were measured using a Dionex Model DX-120 ion chromatography apparatus (Dionex, Sunnyvale, CA) equipped with Dionex AS14-4 and AG 14-4 columns and a conductivity detector. Soluble chemical oxygen demand (SCOD) in the bulk was measured after filtration (0.45 μm) using the dichromate reactor digestion method with colorimetric test kits (Hach-8000).
3.3.7. Micro-profile measurements

The activated sludge flocs were taken from the SBR reactor at the end of the anaerobic stage in the EBPR operation. The microprofile measurements were carried out inside a Faraday cage (Technical Manufacturing Corporation [TMC], Peabody, Massachusetts) to minimize signal noise, as shown in Fig. 3.5(a). An up-flow chamber with laminar flow conditions was used during microprofile measurements in which the floc was kept suspended but stationary while the microelectrode was inserted into the floc (Ploug et al. 1999; Li et al. 2004) (Fig. 3.5(b), Fig. 3.5(c)). The up-flow chamber was mounted under a stereo microscope with a charge-coupled device (CCD) camera (Model JE-3662 HR, Javelin Elec., Torrance, California) situated on a Micro-g series high performance vibration isolation table (63-527-01, TMC, Peabody, Massachusetts) inside the Faraday cage. Feed solution with the same composition as in the anaerobic stage of the operation was continuously flowed using a peristaltic pump (Cole-Parmer, Vernon, IL). Nitrogen gas was injected into the feed tank to establish anaerobic conditions. By controlling the velocity into the up-flow chamber, a parallel and uniform upward flow was achieved, in which a floc can be stabilized in suspension. The water inside the up-flow chamber overflowed evenly through four outlets at the upper part of the chamber, as shown in Figure 3.5(b). A stereomicroscope, a CCD camera, and a color monitor were used to monitor the stabilization of the flocs and the location of the microelectrode tip (Fig. 3.5(a)). Positioning and movement of the microelectrode tip toward the flocs was controlled using a 3-dimensional micromanipulator (model 11N, Narisige, Japan), which was located outside the Faraday cage. The average diameter of the activated sludge flocs for the phosphate concentration microprofile measurement was around 1,000 µm and
microprofiles were recorded at 100 µm intervals into the flocs. A pH meter (model 215, Denver Instruments, Denver, Colorado) was used to obtain potentiometric signals (mV) and a Balance Talk Spreadsheet Logger Transfer Management (Labtronics Inc., Ontario, Canada) spread logger was used to record these electrode responses (mV) continuously for monitoring the phosphate response. A silver/silver chloride milli-electrode (MI-401, Microelectrodes Inc., Bedford, New Hampshire) was used as a reference electrode. A commercial oxygen mini-electrode (OM-4 oxygen sensor, Microelectrodes Inc.) was used to measure the oxygen concentration in the up-flow chamber while monitoring phosphate in the anaerobic phase in the EBPR process. Phosphate concentration in the bulk solution was measured using a Dionex (Sunnyvale, California) Model DX-120 ion chromatography apparatus equipped with Dionex AS14-4 and AG 14-4 columns and a conductivity detector.

3.3.8. Estimation of effective diffusion coefficient

Phosphate concentration microprofiles, which were measured using the phosphate microelectrode, were analyzed to determine the effective diffusion coefficient for phosphate release from the flocs with the same procedures that were originally used to demonstrate dissolved oxygen profiles in a biofilm (Abrahamson et al. 1996; Lewandowski et al. 2003). This procedure has been described in details by Lewandowski (1994). The phosphate microprofile was divided into two parts: inside the activated sludge floc, which provided information about microbial activity, and in the bulk liquid, which provided information about phosphate release from the flocs. If we assume that activated sludge flocs are uniform and that mass transfer is one dimensional, the shape of
the phosphate microprofile inside and outside the floc can be described by the following empirical equation (Abrahamson et al. 1996).

Inside floc,

\[
\frac{C - C_s}{C_f - C_s} = 1 - e^{-A(x-x_s)}
\]  \hspace{1cm} (3.4)

Outside floc (in the bulk),

\[
\frac{C - C_s}{C_s - C_b} = 1 - e^{-B(x-x_s)}
\]  \hspace{1cm} (3.5)

where, \( C \) (mg/L) is the local phosphate concentration, \( C_s \) is the phosphate concentration at the surface of the flocs, \( C_f \) is the phosphate concentration in the activated sludge flocs, \( C_b \) is the phosphate concentration in the bulk, \( x \) (cm) is the distance from a certain point of bulk fluid where the phosphate concentration is constant, \( x_s \) (cm) is the distance between \( x \) and the surface of the flocs, and \( A \) and \( B \) are experimental coefficients determined graphically from the slope of a linearized form of eqs. (3.4) and (3.5).

3.3.9. Molecular Analysis

3.3.9.1. Sampling and extraction of DNA from mixed liquor

Two ml of mixed liquor was obtained from the reactor of the laboratory SBR and was stored at -80°C for 24 hours. Deoxyribonucleic acid (DNA) was extracted from 1 milliliter of thawed mixed liquor from the sample stored at -80°C. Extraction was carried out using combined physical (bead beating) and chemical lyses techniques following the manufacturer’s instructions specified in the MoBio Soil DNA Extraction Kit (MoBio Laboratories Inc., Solana Beach, CA). The quantity of extracted DNA (5 µl of extract in
495 ml of deionized water) was measured using UV spectrophotometry (Genosys Model 10UV/Bis Spec).

### 3.3.9.2. PCR conditions and cloning

Two PCR primers, S-D-Bact-0011-a-S-17; 5’ to 3’ sequence = GTTTGATCCTGGCTC AG, and S-D-Bact-1492-a-A-21; 5’ to 3’ sequence = ACGYTACCTTGTTACGACT T, were used to amplify the 16S rRNA gene from the DNA (Zhang et al. 2006). Amplification was performed with a thermal cycler (Applied Biosystems Model 2400 Thermal Cycler) by using a total of 25 μl of a mixture containing 4 μl genomic DNA extract and 21 μl of master mix. The master mixture consisted of the two primers, PCR buffer, Tag DNA polymerase and MgCl₂. The PCR technique involved denaturing the DNA at 94°C, annealing at 55°C and extension at 72°C. Amplification of PCR products was verified by electrophoresis using 0.5% (w/v) agarose gel stained with ethidium bromide. The fresh PCR product was then cloned using a TOPO TA cloning kit, version K2 (Invitrogen Corp, CA). During this process, PCR product was inserted into the plasmid vector, which contained the antibiotic resistant gene and the lacZ gene, and plasmids were transformed into *E. coli* cells.

### 3.3.9.3. Plasmid DNA extraction and sequencing

Subsequently, *E. coli* cells were grown in a media containing X-gal and kanamycin (Baca-DeLancey et al. 1999). White colonies of *E. coli* were selected and cultured over night prior to extracting one type of plasmid DNA. Plasmid DNA was extracted following the same procedure as explained in the section on sampling and extraction of
DNA. Plasmid DNA was then purified using a QAIprep Spin Miniprep Kit (Qiagen Inc, CA). Sequencing was performed in the DNA Core Facility at the College of Medicine, University of Cincinnati. The nucleotide sequencing data was used to detect phylogenetic affiliation using the Basic Local Alignment Search Tool (BLAST) of the Genbank and European Molecular Biology Laboratory databases.

### 3.3.10. Microscopy

Activated sludge flocs which were taken from the reactor were analyzed by using epifluorescence microscopy (Nikon, Eclipse E600, Melville, NY). The flocs were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (1 mg/L) for 2 minutes and BET 42a (L-Sc-bProt-1027-a-A-17; GCCTCCACTTCGTTT) for 15 minutes. The stained floc samples were rinsed with deionized water to remove the excess dye and rapidly air dried. The stained samples were visualized using a Nikon Eclipse E600 epifluorescence microscope (10 × objective), and images were obtained using a Spot Camera and the Spot Advanced Software.

### 3.4. Results and Discussion

#### 3.4.1. Calibration

Fig. 3.2 shows the finished phosphate microelectrode. Seven different concentrations of standard phosphate solution were prepared with a range of $10^{-5.8}$ to $10^{-2}$ M KH$_2$PO$_4$ for calibration. During EBPR operation in the SBR mode, the pH was approximately 7.5 in the anaerobic phase and 7.5 to 8.3 in the aerobic phase. Therefore, the calibration and characterization of the phosphate microelectrode was conducted in a pH 7.5 buffer.
solution and the pH of the standard solutions was adjusted to 7.5 by adding potassium hydroxide. At pH 7.5, MINEQL+ showed dihydrogen phosphate ion and hydrogen phosphate ion to be the dominant species, as shown in Table 3.1. Fig. 3.6(a) shows that the phosphate microelectrode performed linearly, and exhibited high sensitivity toward these two ions (i.e. dihydrogen phosphate ion and hydrogen phosphate ion) with slopes of 33.1 mV/decade over a wide range of concentrations. The response time during the calibration was less than 1 minute. Fig. 3.6(b) describes the detection range of the phosphate microelectrode which was calibrated several times over two days. Even though a steady state potential during a continuous period of measurement was observed at a low concentration of $10^{-6}$ M, the standard deviations at concentrations below $10^{-6}$ were high at 26.2 (at $10^{-7}$ M) to 56.3 (at $10^{-6}$ M) mV. Therefore, the detection range of the phosphate microelectrode was confined to the range of $10^{-5}$ to $10^{-1}$ M, as shown in Fig. 3.6(b). The detection limit of the phosphate microelectrode was found to be $10^{-5.1}$M ($7.9\times10^{-6}$ M), which was calculated using the value of blank signal (-250mV), standard deviation (56.3) at $10^{-6}$ and the slope of the calibration curve. The detection limit, which was calculated here, is theoretical value based on the experimental data. When we applied the developed phosphate electrode to measure phosphate concentration in the nitrifying biofilm in the simulated drinking water distribution system, we could detect the phosphate up to $2.25\times10^{-6}$ M (0.07mg P/L), as shown in Chapter 7, with highly stable responses (mV).
3.4.2. Stirring effect

The effect of stirring on phosphate measurement was investigated to verify sensitivity and stability of response during flowing conditions at pH 7.5. A stirring plate was used for agitation at 200 rpm and the phosphate concentration in the test solution was $10^{-3}$ M. For *in situ* phosphate measurements, the convection of the bulk solution where the biofilms or activated sludge flocs are immersed can be a critical factor for measurement error. Theoretically, stir rate should have no effect on the recorded potential under potentiometric conditions. With stirring, the response time was shortened and the electrode response value was found to change less than 5 mV, which is within ± 3 % of the linear range of measurement (data was not shown). The response factor (slope) of the calibration curve with stirring was found to maintain its linear relationship with phosphate concentration. It can be concluded that the response was not influenced by stirring.

3.4.3. DO interference

When 0% and 21% DO concentrations were applied to the standard solution, the phosphate electrode response was highly affected by the concentration of DO; the electrode response changed from -580 mV to -330 mV in $10^{-4}$ M standard solution at pH 7.5 (data was not shown). This result indicated that dissolved oxygen has a significant effect on the *in-situ* measurement of phosphate. To provide a more detailed understanding, the DO interference was assessed at eight different DO concentrations ranging from 0% to 14% at 23°C. This DO range was based on the operational results from an EBPR process operating in our lab.
Fig. 3.7 shows changes in the calibration curve with different DO concentrations. The calibration curves show that the DO effect on the measurement of phosphate when the DO is above 2 % is minimal. But it becomes more significant below 2 % DO. The value of the Y-intercept decreased as DO decreased, with the slope ranging from 30.8 - 40.7. These results indicate that oxygen interferes with the binding mechanism between cobalt oxide and phosphate so that the response decreases as dissolved oxygen is increased. Dissolved oxygen concentrations between 0% and 2% have a significant effect on the sensitivity of the phosphate measurement. While DO concentration shifted the Y-intercept, the response (slope) still showed good sensitivity toward phosphate ions. From the results, it is recommended that DO profiles should be measured simultaneously with phosphate in environmental applications, especially microbial flocs in which DO is known to gradually be depleted with depth into the floc (Li et al. 2004).

3.4.4. pH interference

When comparing results at pH values ranging from high (pH 12.0) to low (pH 4.0), a linear calibration was successfully obtained at most pH values. However, the response was poor at the highest pH. Examination using MINEQL+ showed that Co(OH)$_2$ is the dominant species above pH 11.0. It appears, due to formation of the precipitate Co(OH)$_2$ ($K_{sp}$: $1.6 \times 10^{-15}$) at the surface of the cobalt, that the electrode response was decreased, as was reported by Chen et al. (1998). Therefore, the microelectrode is predicted to have selectivity to phosphate ion below pH 11.0. The pH interference was further studied across a narrow pH range from 6.7 to 8.4 to correspond to conditions within the EBPR process (pH 7.2 - 8.4). Fig. 3.8 shows that the phosphate microelectrode has a linear
relationship toward the two phosphate ions (H$_2$PO$_4^-$, HPO$_4^{2-}$) according to the solution pH, with the phosphate microelectrode being slightly more sensitive at higher pH. The proportion of these phosphate ions at each tested pH value is shown in Table 3.1. Typical pH variation across the activated sludge flocs is usually within 0.5 pH units (Li et al. 2004). In this range, the effect of any pH change is minimal, and there is no need to measure pH simultaneously. However, in a case of highly varied pH, it is recommended that the calibration curve should be acquired at the appropriate pH before measuring phosphate.

### 3.4.5. Ion interference

Both indirect and direct methods were used to investigate potential ion interferences. For the indirect method, interfering ions were kept constant, while phosphate ion was varied from $10^{-2}$ to $10^{-5}$ M phosphate. In the direct method, phosphate ion was kept constant, while interfering ions were varied. The four common anions (sulfate, chloride, nitrate, and acetate) in wastewater were tested and the concentrations of these ions were chosen based on a literature review (Wang et al. 2005; Erdal et al. 2003; Yu et al. 2000; Wang et al. 2005) and preliminary experiments, as shown in Table 3.2. The middle concentrations ($9.1 \times 10^{-4}$ M SO$_4^{2-}$, $5.4 \times 10^{-3}$ M Cl$^-$, $5.1 \times 10^{-4}$ M NO$_3^-$, and $2.6 \times 10^{-4}$ M CH$_3$COO$^-$) among three concentrations of each ion in Table 3.2 indicated a typical concentration in a wastewater (Wang et al. 2005).

Fig. 3.9(a) – 3.9(d) show the results of the individual ion interference investigations at pH 7.5. Even though the presence of ions shifted the electrode response toward phosphate ions, ion interference in the typical wastewater at pH 7.5 was demonstrated to
be minor and the microelectrode successfully produced a linear relationship with phosphate ions. Fig. 3.9(e) shows the interference of alkalinity at higher alkalinity in the sample on the electrode performance. Anions such as \( \text{CO}_3^{2-} \) and \( \text{OH}^- \) in solutions which have alkalinity were reported to react with \( \text{Co}^{2+} \) to form precipitates such as \( \text{CoCO}_3 \) (\( K_{sp}: 1.4 \times 10^{-13} \)) and \( \text{Co(OH)}_2 \) (\( K_{sp}: 1.6 \times 10^{-15} \)) (Dean 1995), and another researcher explained that this precipitate of \( \text{CoCO}_3 \) and \( \text{Co(OH)}_2 \) affects the cobalt electrode response (Schecher et al. 2003). From Fig. 3.9(e), alkalinity over 100 mg/L as CaCO\(_3\) was found to be an interference factor due to \( \text{CoCO}_3 \) and \( \text{Co(OH)}_2 \) precipitates formed on the tip surface of the microelectrode. Fig. 3.9(a) - 3.9(e) also indicate the developed microelectrodes using cobalt material responses follow the pattern of \( \text{HPO}_4^{2-} \), \( \text{H}_2\text{PO}_4^- \) > \( \text{HCO}_3^- \) > \( \text{Cl}^- \) > \( \text{SO}_4^{2-} \) > \( \text{NO}_3^- \) > \( \text{CH}_3\text{COO}^- \), and selectivity coefficients (Marco et al. 2003) and ionic strengths in the Table 3.2 support these patterns.

Fig. 3.10 shows the calibration using actual bulk solution used for measuring the microprofiles in the activated sludge flocs, compared to calibrations with and without mixed ions. The bulk solution was taken from the end of the anaerobic phase in the EBPR process, where the COD had been reduced from 216 mg/L to 10 mg/L and phosphate had increased from 3 mg/L as P to 15 mg/L as P due to the phosphate release by the PAOs (Fig. 3.17). Even though the results of calibrations in real wastewater had lower slopes than that in standard solutions, the calibration curves still had good linear relationships with phosphate ions and it was possible to measure the phosphate concentration in the microbial flocs with depth.
3.4.6. Life time and regeneration

For long term stability testing, the microelectrode was immersed in a solution with a high concentration of phosphate (10\(^{-3.8}\) M KH\(_2\)PO\(_4\)). Fig. 3.11 shows the response was relatively stable for a long period (12 days). However, at a low concentration of phosphate, the response was found to change over time. Fig. 3.12 shows electrode response changes and reliabilities for phosphate measurements at several contact times (0 to 10 days) at various phosphate concentrations. The freshly fabricated microelectrode was contacted with phosphate in a 10\(^{-4}\) M phosphate standard solution at pH 7.5, and the calibration curve was determined after 0, 2, 4, 6, and 10 days. Over longer contact times, the electrode response at the high concentration of 10\(^{-3.8}\) M phosphate did not change much, but a larger difference was observed in the potential at lower phosphate concentrations. It appears that cobalt phosphate precipitation (Co\(_3\)(PO\(_4\))\(_2\)) from the reaction shown in Eq.(3.2) could decrease the response at low concentrations of phosphate. Results from SEM with EDS analysis showed that the phosphate weight percentage at the microelectrode surface area increased after the phosphate reaction due to the formation of cobalt phosphate. With additional contact time, a recess at the tip of the microelectrode developed due to corrosion. Fig. 3.13(a) shows a recess length of 20 \(\mu\)m was generated at the 10 \(\mu\)m diameter tip of the phosphate microelectrode after 12 days of exposure to phosphate ion.

From these results, the cobalt phosphate precipitation and diffusional reactions resulting from a recess generation could increase the variation of response between certain phosphate concentrations and make more detailed quantitative analyses for phosphate possible. Therefore, generation of a recess manually by etching before
measurement is recommended to increase the response range. These results also indicate that shelf life time is not affected by the storage period, but is a function of the exposure time. Fig. 3.13(b) shows that the microelectrode had a 20 μm tip diameter after rebeveling and recovered its original characteristics, with a similar slope in the calibration curve as shown in Fig. 3.14.

3.4.7. Corrosion reaction characteristics

Fig. 3.15 shows the results of CV testing, which was conducted to elucidate the recess generation mechanism. The peak current at 200 mV increased with phosphate concentration, indicating that phosphate was involved in the electrochemical oxidation process. However, reduction on the cathodic side was less than oxidation at the anode. This showed that the response to phosphate is not based on any ion-selective equilibrium binding mechanism, but rather on changes in the oxidation corrosion potential of the wire due to cobalt ion binding to phosphate ions. Therefore, it appears that the recess was generated due to corrosion after response to the phosphate ion.

SEM evaluations with EDS were carried out to analyze the microelectrode surface layer before and after the corrosion reaction between phosphate and cobalt metal. Fig. 3.16 shows SEM images before and after reaction of cobalt with phosphate. The microelectrode had a clear surface before the phosphate reaction (Fig. 3.16(a)). After reaction with phosphate ion, cobalt corrosion was observed, as shown in Fig. 3.16(b). This result supports the theory that phosphate response at the cobalt surface was based on a change in oxidation corrosion potential, and corrosion at the surface of the microelectrode can explain the possible disintegration of cobalt metal seen in Fig. 3.16(b).
when the current was employed, even though the soluble cobalt concentration in the solution was not detected; this was probably due to the very low concentration, below the detection limit of FAAS.

3.4.8. In-situ monitoring of phosphate in the microbial flocs

The SBR reactor for the EBPR process was operated over 6 months with 3 cycles/day and showed high COD and phosphorus removal (i.e. 5 - 10 mg/L COD effluent concentration from 200 - 250 mg/L of COD influent concentration, and an average 0.6 mg/L of phosphorus effluent concentration from 8 - 10 mg/L of phosphorus influent concentration). Fig. 3.17 shows the one cycle batch experiment results. This time-track analysis was conducted before microprofile measurement to investigate the operation of the EBPR process and select appropriate times for phosphate microprofiling in the anaerobic phase. From the results, it can be observed that PAOs in the reactor released phosphorus and increased the 3.0 mg/L of influent phosphate to 15.0 mg/L at the end of the anaerobic phase, and then performed luxury phosphorus uptake in the aerobic phase. At the end of the aerobic phase, the phosphate concentration was down to 2.0 mg/L. The phosphorus concentration in the bulk solution was increased in the anaerobic phase (AN) and decreased during the aerobic phase (OX) due to the PAOs’ unique microbial activities (Rittmann et al. 2001). The dissolved oxygen (DO) concentration was zero during the anaerobic phase, while in the aerobic phase, the DO concentration was maintained above 2.0 mg/L. From the Fig. 3.17, there was little change in the phosphate concentration after 1 hour of phosphate release by PAOs in anaerobic stage. If it is assumed that the phosphate was fully released during the anaerobic condition and the
phosphate was not consumed by microbes but transferred only by diffusion from the flocs to the bulk, the effective diffusion coefficient for phosphate release can be determined from the measured phosphate microprofiles. Consequently, the time for these phosphate microprofiles measurements were set up at the end of the anaerobic phase, where the dissolved oxygen was nearly zero, and thus caused no DO interference with the selectivity for phosphate.

As discussed earlier, it has been reported that the cobalt-based phosphate microelectrode is responsive to oxygen concentration in the solution (Meruva et al. 1996; Lee et al. 2008; Xiao et al. 1995). If the oxygen remained inside the activated sludge floc, during the anaerobic phase, even though the bulk DO concentration was zero as shown in Fig. 3.17, PAOs would have consumed it due to the aerobic microbial activity, and conversely a little phosphate uptake would have been observed in the reactor. However, the phosphate was continuously and increasingly released from the flocs during the anaerobic phase and no aerobic microbial activity, including nitrification, was observed from Fig. 3.17. In addition, previous research about DO microprofiling indicated that, due to microbial oxygen utilization, the aerobic region in the activated sludge floc was limited to the surface of the flocs (100 – 200 µm) and the DO concentration was decreased according to the depth, even at a bulk DO of 4.0 mg/L (Li et al. 2004). Therefore, it was concluded that there was no oxygen available in or out of the flocs during the anaerobic phase, and phosphate measurement was not affected by oxygen in this experiment.

The locations of the microelectrode measurements were carefully selected to minimize the effect of floc heterogeneity and irregular surface structure. For example,
well-rounded flocs between 1,000 – 1,200 μm diameter were selected in this study. The flocs for phosphate measurement were taken at the end of the anaerobic stage of the EBPR process. Therefore, it was assumed that enough phosphorus release had occurred throughout the reactor, achieving a pseudo-steady-state condition, and these concentration profiles did not change, in relation to the time of microbial growth. The measurement of profiles was taken within 30 minutes. The microelectrode readings were recorded at 100 μm intervals.

Three to five activated sludge floc samples were tested and well-defined phosphate profiles across the flocs were observed. All measured phosphate microprofiles showed higher phosphate concentrations within flocs, compared to the bulk phosphate concentration. A representative phosphate concentration microprofile is shown in Fig. 3.18. The diameter of the floc was approximately 1,000 μm and the center of the floc is depicted at a depth of 0 μm. The points are mean values of triplicates measurements with standard deviations between 0.13 and 0.76. The results show that the monitoring of phosphate was successful in defining spatial distributions of phosphate inside microbial flocs without destroying their structures. Fig. 3.19 shows a photograph of the penetration of the phosphate microelectrode into the floc. The phosphate concentration in the bulk at the end of the anaerobic stage was about 15.1 mg/L as P where phosphate release was at about two-fold the amount of the inflow concentration (7.5 mg/L as P). As the microelectrode penetrated the flocs, the phosphate concentration of 15.1 mg/L in the bulk solution increased as the floc was penetrated toward the center, finally slowing as it reached a peak value of about 20.4 mg/L at the floc center. Wang et al. (2005) indicated that the higher phosphate concentration in the floc center suggests that there is a higher
density of PAOs at the center of the floc than at the edge of the floc using microelectrodes; this was further confirmed using fluorescent in situ hybridization techniques. From these results, it seems that the phosphate concentration distribution in the flocs showed that the PAOs grow evenly in the activated sludge flocs, and the highest phosphate value in the floc indicated the highest density of PAOs in the floc. The diffusion boundary layer, in which the phosphate concentration started to change near the flocs, was defined as 100 μm thick.

3.4.9. Effective diffusion coefficient for phosphate release

The effective diffusion coefficient of phosphate release was determined from the measured phosphate concentration microprofiles (Fig. 3.18). The microprofile was reformatted as shown in Fig. 3.20. Two inflection points, at 0.025 cm and at 0.135 cm, of the profile indicate the surfaces of the floc. Two parts of the data set were segmented with location; one inside the floc and the other outside the floc. \( x_s \) is 0.025 cm and at this point, and the phosphate concentration \( (C_s) \) was 16.9 mg/L. Inside the floc, the phosphate concentration \( (C_f) \) was 20.4 mg/L and the bulk phosphate concentration \( (C_b) \) was 15.1 mg/L. From the measured microprofile, all the values in these equations, except coefficients A and B, were obtained, and these equations were transformed to a linearized form to find these coefficients.

Inside the floc, equation (3.4) can be linearized to find coefficient A from the experimental data.

\[
\ln \left(1 - \frac{C - C_s}{C_f - C_s}\right) = -A(x - x_s) \tag{3.6}
\]
Here, $C_f$ is 20.4 mg/L as P, $C_s$ is 16.9 mg/L as P, and $x_s$ is 0.025 cm (Fig. 3.20). Coefficient $A$, calculated as the slope of the line shown in Fig. 3.21 determined by presenting the data in coordinates $(x-x_s)$ versus $\ln(1-(C-C_S)/C_f-C))$, equals $-62.0 \text{ cm}^{-1}$. The slope in Fig. 3.21 is $A = 62.0 \text{ cm}^{-1}$.

The differentiated form of equation (3.4) becomes equation (3.7).

$$\left(\frac{dC}{dx}\right)_{f,X_s} = A(C_f - C_s) \quad (3.7)$$

Therefore, $(dC/dx)_{f,X_s}$ calculated from equation (3.7) was 265.4 mg/L-cm and this value indicates the phosphate concentration gradient inside floc.

In the bulk solution (outside the floc), equation (3.5) can be linearized to determine coefficient $B$ from the experimental data.

$$\ln\left(1-\frac{C-C_S}{C_S-C_b}\right) = -B(x-x_s) \quad (3.8)$$

Here, $C_b$ is 15.1 mg/L as P, which is the phosphate concentration in the bulk liquid. Coefficient $B$, calculated as the slope of the line shown in Fig. 3.22 determined by presenting the data in coordinates $(x-x_s)$ versus $\ln(1-(C-C_S)/C_f-C))$, equals $-45.2 \text{ cm}^{-1}$. The slope in Fig. 3.22 is $B = 45.2 \text{ cm}^{-1}$.

The differentiated form of equation (3.5) becomes equation (3.9).

$$\left(\frac{dC}{dx}\right)_{W,X_s} = B(C_S - C_b) \quad (3.9)$$

Therefore, $(dC/dx)_{W,X_s}$, phosphate concentration gradient outside floc was 81.8 mg/L-cm.
If it is assumed that the flux rate \( J_{f,Xs} \) of phosphate diffusion from the center of the activated sludge floc to the surface of the floc is equal to the phosphate flux \( J_{W,Xs} \) across the stagnant layer, then:

\[
J_{f,Xs} = J_{W,Xs} = D_f \left( \frac{dC}{dx} \right)_{f,Xs} = D_w \left( \frac{dC}{dx} \right)_{w,Xs}
\]  \( (3.10) \)

Here, \( D_f \) is the effective diffusivity of phosphate in the activated sludge floc and \( D_w \) is the diffusivity of phosphate in the water. The phosphate ion diffusivity \( (D_w) \) in water is \( 1.25 \times 10^{-6} \) cm\(^2\)/s at 20ºC (Krom and Berner 1980). Therefore, \( D_f \) was calculated to be \( 3.33 \times 10^{-7} \) cm\(^2\)/s, which was the average value of triplicate measurements. The relative phosphate effective diffusivity \( = \frac{\text{effective diffusion coefficient}}{\text{diffusivity in water}} \) or \( \frac{3.33 \times 10^{-7}}{1.25 \times 10^{-6}} \) was 0.27. In this experiment, the system was operated in a SBR mode to simulate EBPR process. Therefore, the value of \( D_f \) \( (3.33 \times 10^{-7} \) cm\(^2\)/s) can be different with the value which can be obtained from the anaerobic portion of an EBPR oxidation ditch or other phosphate removal processes. The effective diffusion coefficient which was determined in this study might be the minimum phosphate diffusion rate of phosphate release which PAOs can have in this experimental process, because it was measured at the end of the anaerobic phase where it was expected that the phosphate was fully released from the activated sludge flocs.

### 3.4.10. Sequencing and FISH analysis

11 sequencing results were obtained and each result has approximately 860 bp of nucleotide sequence. The sequences of the results have been deposited at GenBank under the project accessions GU065721-GU065731. The sequencing results were compared to
the data in the molecular database using the basic local alignment search tool (BLAST). Best matches in Table 3.3 were selected with over 90% of Max ident in BLAST. These results reveal that there are two species of PAOs in the reactor that are similar to previous studies, as shown in Table 3.3. There are numerous cases in which bacteria with identical 16S rRNA gene sequences have very different physiological activity (e.g. perchlorate-respiring bacteria). However, if we assume that the reactor has worked properly for phosphorus removal based on the operation monitoring (i.e. phosphate uptake and release in bulk solution), it appears that the identified organisms, including *Sinorhizobium* sp., would be mainly PAOs and this confirms that the measured phosphate concentration microprofiles showed PAOs activity. From the FISH results, it was found that the sample from the reactor also contained general wastewater bacteria, as shown in Figure 3.23.

### 3.5. Conclusions

The performance of a new cobalt-based phosphate microelectrode was investigated under different conditions (e.g., pH, DO, with interfering ions, and with different alkalinities). The developed microelectrode performed linearly and exhibited high sensitivity toward phosphate ions. The response time during the evaluation was less than 1 minute. The detection range of the phosphate microelectrode was in the range of $10^{-5}$ to $10^{-1}$ M and the detection limit of the phosphate microelectrode was found to be $7.9 \times 10^{-6}$ M. The electrode response was not influenced by stirring. While DO was found to be a factor during calibration, the response still showed good sensitivity toward phosphate ions. DO profile measurements along with phosphate profiles were suggested for more precise phosphate analyses. Ion interference experimental results showed alkalinity
above 100 mg/L as CaCO$_3$ in the solution may be a limiting factor for measurement of phosphate. The recess at the tip of the microelectrodes due to corrosion and cobalt phosphate precipitation generated from the reaction between cobalt oxide and phosphate explained the decrease in response at low concentrations of phosphate. However, this condition made it possible to measure phosphate more quantitatively. For better results, generation of a recess manually before measurement by the etching process could possibly increase the response range. Phosphate concentration microprofiles were measured within activated sludge flocs in the EBPR process using a developed phosphate microelectrode. PAOs in activated sludge flocs, that are responsible for a well-operated EBPR process, were indentified using clone library analysis, confirming that the measured microprofiles showed PAO activity. The results showed that the monitoring of phosphate was successfully conducted and could define spatial distributions of phosphate inside microbial flocs without destroying their structures. From the microprofiles, the phosphate concentrations were shown to increase as the depth increased to the center of flocs, and they reached a constant high value at the floc center. If it is assumed that the phosphate is not consumed by microbes and is only transferred from the flocs to the bulk by diffusion during anaerobic conditions because of phosphate release microbial activity, the effective diffusion coefficient ($D_f$) for phosphate release within the flocs was calculated to be $3.33 \times 10^{-7}$ cm$^2$/s at the end of the anaerobic phase of the EBPR process. The stratification of the flocs in the microprofiles indicated that the PAOs were probably distributed fairly evenly throughout the flocs. These results provide valuable information on the microbial activity occurring inside the floc in the EBPR process. Overall, this full characterization and successful application showed that the cobalt-based phosphate
microelectrode could be a useful tool for in situ measurement of phosphate in various environmental samples, including within microbial flocs. In the future, comparison analyses of the phosphate removal process is expected between biofilm and suspended activated sludge flocs to validate the role of PAOs combined with other microelectrode sensors for pH, oxidation-reduction potential (ORP), dissolved oxygen (DO), phosphate, ammonia and nitrate profiling. These micro-profiles, combined with other constituent profiles including pH, DO, ammonia, and ORP, in the flocs can be used to elucidate the dynamic activity of microbial processes in the different types of EBPR process and can be valuable for designing operating systems or modeling efforts for biological nutrient treatment.
Figure 3.1 Poly-phosphate accumulating organisms (PAOs) in the enhanced biological phosphate removal (EBPR) process (Grady et al. 1999).

Figure 3.2 Schematic diagram of the phosphate microelectrode based on cobalt wire with small tip diameter (5-10µm).
Figure 3.3  SEM image of tip: well exposed cobalt wire with a glass body

Figure 3.4  Experimental setup for characterization and evaluation.
(a) Microprofiling experimental setup

(b) Schematic description of the microprofiling in up-flow chamber
(c) Microprofile measurement using phosphate microelectrode

Figure 3.5. Experimental setup for the microprofile measurements.

Table 3.1 Phosphate species$^a$ at different pH values.

<table>
<thead>
<tr>
<th>pH</th>
<th>$\text{H}_2\text{PO}_4^-$</th>
<th>$\text{HPO}_4^{2-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.7</td>
<td>73.7 %</td>
<td>26.3 %</td>
</tr>
<tr>
<td>7.2</td>
<td>47.1 %</td>
<td>52.9 %</td>
</tr>
<tr>
<td>7.6</td>
<td>31.0 %</td>
<td>69.0 %</td>
</tr>
<tr>
<td>8.4</td>
<td>0.4 %</td>
<td>99.6 %</td>
</tr>
</tbody>
</table>

$^a$: based on MINEQL+ analysis
Figure 3.6  Phosphate microelectrode calibration at pH 7.5 and ambient oxygen levels.
Figure 3.7  Dissolved oxygen interference with the phosphate measurement at pH 7.5.

Figure 3.8  pH interference of the phosphate microelectrode with varied pH at ambient oxygen levels.
Table 3.2  Ion concentration range, selectivity coefficient and ionic strength for ion interference evaluation.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Reagent</th>
<th>Range for measurement (M)</th>
<th>Selectivity Coefficient[11]</th>
<th>Ionic Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO₄²⁻</td>
<td>K₂SO₄</td>
<td>9.1×10⁻³ 9.1×10⁻⁴ 9.1×10⁻⁵</td>
<td>1×10⁻³</td>
<td>1.82×10⁻³</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>KCl</td>
<td>5.4×10⁻² 5.4×10⁻³ 5.4×10⁻⁴</td>
<td>2×10⁻³</td>
<td>2.68×10⁻³</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>NaNO₃</td>
<td>5.1×10⁻³ 5.1×10⁻⁴ 5.1×10⁻⁵</td>
<td>8×10⁻⁴</td>
<td>0.25×10⁻³</td>
</tr>
<tr>
<td>CH₃COO⁻</td>
<td>CH₃COONa</td>
<td>2.6×10⁻³ 2.6×10⁻⁴ 2.6×10⁻⁵</td>
<td>1×10⁻³</td>
<td>0.12×10⁻⁴</td>
</tr>
</tbody>
</table>
b. Chlorine ion

- Y = -31.0X - 567.0
  - R² = 0.99
- Y = -21.3X - 544.9
  - R² = 0.99
- Y = -10.4X - 514.4
  - R² = 0.87

\[\log[H_{3-x}PO_4^{-x}]\ (M)\]

5.4 × 10⁻² M
5.4 × 10⁻³ M
5.4 × 10⁻⁴ M

C. Nitrate

- Y = -37.1X - 579.5
  - R² = 0.97
- Y = -29.1X - 561.6
  - R² = 0.99
- Y = -20.0X - 543.0
  - R² = 0.98

\[\log[H_{3-x}PO_4^{-x}]\ (M)\]

5.1 × 10⁻³ M
5.1 × 10⁻⁴ M
5.1 × 10⁻⁵ M
Figure 3.9 Evaluation of the selectivity of the phosphate microelectrode against major anions (a) Sulfate ion; (b) Chlorine ion; (c) Nitrate; (d) Acetate ion; (e) Alkalinity at pH 7.5 and ambient oxygen levels.
Figure 3.10  Comparison of calibration in pH 7.5 standard phosphate solution with mixed ions and without ions in bulk solution in anaerobic phase of EBPR process.

Figure 3.11  Performance of the phosphate microelectrode during 12 days measurement in $10^{-3.8}$ M KH$_2$PO$_4$ standard solution at pH 7.5.
Figure 3.12 Evaluation of the relationship between contact time and response change at pH 7.5 and ambient oxygen levels.

Figure 3.13 (a) Microelectrode tip (10 μm diameter) with recess (20 μm long) after 12 days exposure to phosphate; (b) Microelectrode tip (20 μm diameter) after re-beveling.
Figure 3.14 Evaluation of regeneration through tip re-beveling at pH 7.5 and ambient oxygen levels.
Figure 3.15 Cyclic voltammograms of phosphate microelectrode in 0.05M KHP Buffer at pH 4.0 with varying total phosphate concentrations. (a) 0 M phosphate (0.05M KHP buffer solution); (b) $10^{-5}$ M phosphate; (c) $10^{-4}$ M phosphate; (d) $10^{-3}$ M phosphate; (e) $10^{-2}$ M phosphate.

Figure 3.16 SEM images of the surface of the phosphate microelectrode tip. (a) Before reaction with phosphate; (b) after reaction with phosphate.
Figure 3.17 EBPR process operation results during the experiment (AN: Anaerobic phase, OX: aerobic phase).
Figure 3.18  Typical micro-profile of Phosphate in flocs from EBPR process at the end of the anaerobic phase. Floc diameter was about 1,000 μm and the center of the floc is depicted as a depth of 0 μm.

Figure 3.19  Penetration of microelectrode through the floc during microprofiling.
Figure 3.20 Reformatting of phosphate concentration microprofile for kinetic study.
Figure 3.21 \(\ln\left(1 - \frac{(C - C_s)}{C_r - C_s}\right)\) versus \((x - x_s)\) inside activated sludge flocs. The slope gives the coefficient \(A\) in equation (3).

Figure 3.22 \(\ln\left(1 - \frac{(C_s - C_b)}{C_s - C_b}\right)\) versus \((x - x_s)\) in the bulk. The slope gives the coefficient \(B\) in equation (5).
Figure 3.23 Simultaneous fluorescent *in situ* hybridization of activated sludge flocs with Cy3-labeled probe (BET 42a) (red color in picture) and universal probe (DAPI) (blue color in picture).

Table 3.3  Best matches for sequencing results

<table>
<thead>
<tr>
<th>Organism</th>
<th>Possible Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sinorhizobium</em></td>
<td>Phosphorus removal <em>(Liu et al., 2001; Dabert et al., 2001)</em>,</td>
</tr>
<tr>
<td></td>
<td>Sulfate-deducing <em>(Labrenz et al., 2004)</em></td>
</tr>
<tr>
<td><em>Zoogloea</em></td>
<td>Nitrogen-fixing <em>(Xie et al., 2006)</em></td>
</tr>
<tr>
<td><em>Thauera</em></td>
<td>Phosphorus removal <em>(Zilles et al., 2002)</em>,</td>
</tr>
<tr>
<td></td>
<td>Aromatic compound removal <em>(Mechichi et al., 2002)</em>,</td>
</tr>
<tr>
<td></td>
<td>Denitrifying <em>(Liu et al., 2006)</em></td>
</tr>
</tbody>
</table>
Chapter 4. Chlorine Microelectrode for Measuring Monochloramine within a Drinking Water Distribution System Biofilm

4.1. Abstract

Chlorine microelectrodes with tip sizes of 5-15 μm were developed and used to measure biofilm monochloramine penetration profiles. The chlorine microelectrode showed response to total chlorine including free chlorine, monochloramine, and dichloramine under various conditions. Where monochloramine dominates the system at pH 8.0 and 4:1 Cl₂:N, the chlorine microelectrode had sensitivity toward monochloramine concentrations (0.1 to 30.0 mg Cl₂/L) at an applied potential of +550 mV (Ag/AgCl reference electrode) at 23°C, and it was independent of pH interference at typical drinking water pH (6-9). A linear relationship existed with monochloramine concentration for a given dissolved oxygen (DO) concentration, even at high DO concentrations. However, a 3-D (response vs. DO vs. monochloramine concentration) calibration curve was constructed because the electrode response shifted with DO changes. The chlorine microelectrode successfully measured monochloramine concentration microprofiles with high spatial resolution within a biofilm. Ultimately, this research will lead to the in-situ application of chlorine microelectrodes for measurement of monochloramine within a chloraminated drinking water system biofilm and, along with other microelectrodes (e.g., ammonia, pH, DO), will aid in elucidating nitrification phenomena occurring in the biofilm.
4.2. Introduction

As a result of the implementation of the Stage 1 and Stage 2 Disinfectants and Disinfection Byproduct Rules, chloramine use as a secondary disinfectant in the United States is predicted to increase to 57% for all surface water and 7% for all ground water treatment systems (USEPA, 2005). A recent survey suggests that an additional 12% of drinking water utilities are contemplating a switch to monochloramine in the future to comply with these rules (Seidel et al. 2005; Wolfe et al. 1984; Regan et al. 2007). However, for utilities that use monochloramine or have source water ammonia, nitrification in distribution systems is an undesirable side effect of chloramine use that may result in water quality degradation and subsequent non-compliance with existing regulations. Based on utility surveys, 30-63% of utilities have experienced nitrification episodes after switching to chloramines (Seidel et al. 2005). Our understanding of distribution system nitrification is incomplete and micro-scale biofilm research on nitrification has been limited due to the lack of necessary tools. To elucidate nitrification phenomena within distribution system biofilm, microelectrode techniques are needed to profile the chemical constituents that lead to nitrifier growth and inactivation. In the current research, chlorine microelectrodes based on platinum wire were fabricated and evaluated for various conditions, and then applied for use in in-vivo environmental analysis of monochloramine concentrations in biofilm. Several researchers have reported the electrochemical behavior of monochloramine sensors using a platinum or gold disk
(or electrode) with a flow injection technique (Davies 1985). Davies (1985) and Tsaousis (1985) indicated that the anodic reaction of monochloramine with gold and/or platinum at an applied potential of +800 mV (saturated calomel electrode (SCE) reference) was observed with high sensitivity compared with hypochlorous acid. Davies (1985) indicated that the anodic reaction of monochloramine with platinum at an applied potential of +800 mV (SCE reference) was observed with high sensitivity compared with hypochlorous acid. Tsaousis (1985) applied this anodic reaction to the gold electrode at an applied potential of +800 mV (SCE reference) and measured monochloramine with high sensitivity. However, these electrodes are too large (e.g. 0.41 mm diameter platinum wire) to apply at the small scale (~10 μm), and there is currently no commercial or developed chloramine microelectrode for application to biological analyses, including direct measurement of biofilm monochloramine penetration.

This study developed chlorine microelectrodes and investigated their biological applicability for determining biofilm monochloramine profiles. The chlorine microelectrodes have a small tip (5-15 μm), allowing measurement in biofilms without disrupting the biofilm structure. With this microelectrode, the chlorine species which the chlorine microelectrode measures in the bulk were investigated under various conditions. Electro-analysis experiments using Cyclic Voltammetry (CV) were conducted for microelectrode characterization to determine the appropriate applied potential for monochloramine measurement. Possible effects of pH, DO, various chlorine to nitrogen (Cl₂:N) mass ratios, and various media on microelectrode performance for monochloramine measurements were tested, allowing evaluation of the microelectrode’s application to various environmental conditions. Finally, the chlorine microelectrode was
used to measure monochloramine microprofiles within biofilm. This research will lead to a better understanding of the chlorine microelectrode for in-situ application for measuring monochloramine within a chloraminated drinking water system biofilm, and along with other microelectrodes (e.g., ammonia, pH, DO), it will help elucidate the biofilm nitrification phenomena.

4.3. Materials and Methods

4.3.1. Fabrication of the chlorine microelectrode

The fabricated chlorine microelectrode was patterned after a previously described chlorine microelectrode (de Beer et al. 1994). The platinum (Pt) wire (0.127 mm diameter, 99.99% purity, Aldrich Chemical Co.) was cut into 4 - 5 cm length sections. The tip of the Pt wire was etched in a 6 M potassium cyanide (KCN) solution with a +5 V potential. After pulling a lead glass micropipette (O.D.: 1.5 mm, I.D.: 0.75 mm, 15 cm length, World Precision Instruments), the etched Pt wire was sealed in a glass capillary as described previously (Lee et al. 2009; Bishop et al. 1999; Yu et al. 2000; Wang et al. 2005). Subsequently, the sealed tip was beveled to expose the Pt surface, and the glass was re-sealed by heating the tip. Finally, the Pt tip was recessed 5 - 6 µm by etching in a 6 M KCN solution for 1 - 2 seconds and coated with 10 % (wt/vol) cellulose acetate in acetone for 30 sec (Fig. 4.1(a)). The tip diameters of the resulting microelectrodes were 5 - 15 µm.
4.3.2. Calibration and characterization of the chlorine microelectrode

CV tests were performed to determine the appropriate applied potential for monochloramine measurement using a Potentiostat (PalmSens Electrochemical Instruments, BV-2004) and pocket PC (PalmScan vs.1.39) for sensing and data analysis. An Ag/AgCl reference electrode was connected with a Pt mesh as the auxiliary electrode and with the chlorine microelectrode as the working electrode in a 50 mM phosphate buffer (PB) (pH 8.0). Monochloramine concentrations applied to CV tests were 9.8 and 40.8 mg/L as Cl₂. Snoeyink (1980) indicated that the rate of monochloramine formation is fast in the pH range of 7.5 to 9.3; the optimum conditions for the formation of monochloramine are pH 8.3, 25°C, and a 4:1 to 5:1 Cl₂:N mass ratio (Kirmeyer et al. 2004). Deoxygenation was performed before the test with nitrogen (N₂) gas to eliminate any potential oxygen interference. During the test, the solution was stirred and scanned at a rate of 100 mV/s over a potential range of -1,000 to +1,000 mV in 20 mV steps.

To prevent electromagnetic interference, all experiments for determining microelectrode performance were conducted inside a Faraday cage and at 23°C. The output picoampere current signal was measured using a Chemical Microsensor II (Diamond General Corp., Product No 1231) potentiostat. Different monochloramine concentrations in the range of 0.1-30.0 mg/L as Cl₂ were used for calibration by adding the concentrated monochloramine stock solution to the test sample with a known volume. To prepare the monochloramine stock solution, chlorine was diluted from a sodium hypochlorite solution (5% available Cl₂, Ricca Chemical Co.) with pH 8.0 phosphate buffer (PB), and then the calculated amount of ammonia nitrogen was added using a 100 mg N/L ammonia stock solution adjusted to pH 8.0. The resulting stock solution
contained 108 mg Cl₂/L and 27.0 mg N/L, resulting in a 4:1 Cl₂:N mass ratio. During the experiment, the monochloramine solution was kept in an ice bath and in the dark. In the experiments using various Cl₂:N ratios (3:1-5:1), the ammonia-nitrogen concentration was adjusted as required (e.g. 36.0 mg/L as N for 3:1, 21.6 mg/L as N for 5:1).

Purging with N₂ gas removed potential dissolved oxygen (DO) interference during the experiments, except in those experiments investigating DO interference. The effect of DO was evaluated in the range of 0-21% DO (by weight) in the same 50 mM, pH 8.0 PB. A commercial oxygen milli-electrode (MI-730, Microelectrodes Inc.) was used to verify the concentration of DO during the test. The experiments for evaluation of pH effect also were conducted in 50 mM PB and at pH 6, 7, 8, and 9 with a monochloramine stock solution containing 108 mg Cl₂/L and 27.0 mg N/L at pH 8.0. For the chlorine to nitrogen mass ratio experiments, chlorine concentration was fixed at 108 mg Cl₂/L and ammonia was adjusted to the desired Cl₂:N ratios (3:1-5:1). The 50 mM PB solution was prepared by mixing the proper amount of 50 mM KH₂PO₄ and 50 mM K₂HPO₄, and adding sodium hydroxide (NaOH) or sulfuric acid (H₂SO₄) to obtain the specific pH value. Then, the stock monochloramine solution, prepared at pH 8.0, was added to each desired pH PB solution. Media effect was investigated in the following solutions: dechlorinated tap water, 50 mM PB, 5 mM PB, and 5 mM carbonate buffer (high alkalinity condition) with a 4:1 Cl₂:N mass ratio and pH 8.0. Because most conditions tested were at pH 8.0 under known monochloramine conditions, most of the figures in this chapter show calculated monochloramine concentrations. In any cases where conditions other than monochloramine were expected, measured monochloramine concentrations are indicated in the figures.
For experiments on measurement of breakpoint chlorination and chlorine or monochloramine residuals, stock ammonia solution (100 mg/L as N) was added to each 50 mL of 5 mM PB solution (pH 7.0) to achieve an ammonia concentration of 1.0 mg/L as N. Then, standard free chlorine stock solution (125 mg/L as Cl₂) was adjusted in each solution to make chlorine to ammonia-nitrogen ratios of 2.0 - 16.0 at regular intervals of 2.0 (Shang et al. 1999). Concentrations of total chlorine or monochloramine residuals were measured 1 hour after chlorine addition using colorimetric test kits (Total chlorine: Hach-8167; Monochloramine: Hach-10200) and a DR/2010 spectrophotometer (Hach Co.). The microelectrode surface was analyzed using scanning electron microscopy (XL 30 ESEM-FEG, Philips XL Series with EDAX Genesis XMS 2000 Energy Dispersive System, 30 KeV of beam energy) to investigate the oxide layer formed on the platinum.

4.3.3. Biofilm growth and microelectrode measurements

A multi-species biofilm was developed on glass slides submerged in a 4-L Sequencing Batch Reactor (SBR) that was part of a biofilm reactor (Lee et al. 2009). The biofilm was grown under high substrate concentrations for easier control over biofilm thickness and cell density, leading to better reproducibility. The synthetic feed solution contained 384.4 mg/L CH₃COONa, 33.7 mg/L KH₂PO₄, 93.3 mg/L (NH₄)₂SO₄ and trace minerals (Lee et al. 2009). This system resulted in a thick biofilm (over 1,000 µm) to evaluate the microelectrode operation in subsequent monochloramine microprofiling. While this biofilm was grown under conditions that do not closely represent drinking water biofilm, future biofilm microprofiling experiments will use biofilm grown under more representative conditions. A glass slide holding a biofilm sample was taken from
the reactor and was placed into a specially designed flow chamber (4.1(b)). The chlorine microelectrode and an Ag/AgCl reference electrode were placed inside the bulk solution of the flow chamber. Fluid with the target monochloramine concentration was flowed across the flow chamber until the bulk monochloramine concentration became stable. The flow rate of the solution into the flow chamber was maintained at 15 mL/min. Then, a microelectrode was mounted under a microscope and its movement was controlled using a three-dimensional micromanipulator (Model 11N, Narisige). During the measurement, N₂ gas, which has no effect on Cl₂ or ammonia concentration, was used to purge dissolved oxygen (DO) to minimize possible interferences. Microprofile measurements were carried out in a 50 mM PB at pH 8.0, where monochloramine is the dominant chlorine compound in the bulk solution (Jafvert et al. 1992; Vikesland et al. 2001). Chlorine and ammonia were supplemented to produce the target monochloramine concentration of 10 mg Cl₂/L (4:1 Cl₂:N). Monochloramine profiles were measured at an applied potential of +550 mV in the flow chamber after 25 and 45 minutes of contact time. The signal was recorded using a Chemical Microsensor II (Diamond General Co.). The biofilm thickness was measured with a second microelectrode. When the second microelectrode tip reached the biofilm surface, the biofilm surface was detected by visibly observing a small movement of the biofilm. Then, this microelectrode was slowly inserted into the biofilm; when the microelectrode reached the substratum, the glass and wire shaft of the electrode would bend slightly. This point was marked as the base of the biofilm. Monochloramine measurements were performed vertically at 50 µm intervals in the biofilm using a micromanipulator under a stereo microscope with a CCD camera (Model JE-3662 HR, Javelin Elec.) inside a Faraday cage. Detailed information
regarding the microelectrode experiment setup can be found elsewhere (Li et al. 2004). As a result, each complete microprofile measurement was recorded in approximately 6 minutes for these thick biofilms. Before and after microprofile measurements, the monochloramine chlorine concentration in the bulk fluid was measured using a colorimetric test kit (Hach-10200) and a DR/2010 spectrophotometer (Hach Co.).

4.4. Results and Discussion

4.4.1. Performance evaluation of the chlorine microelectrode for monochloramine measurement

4.4.1.1. Development of the microelectrode and operating conditions

Chlorine microelectrodes with 5-15 µm tip diameters were fabricated using platinum wire. Initial experiments were conducted under conditions where monochloramine is the dominant chlorine species present (pH 8.0 and 4:1 Cl2:N) (Jafvert et al 1992; Vikesland et al 2001). Platinum is the most widely used electrode material for coulometric, potentiometric, and amperometric techniques (Davies 1985), but our understanding of chloramine electrochemical behavior is not well understood (Piela et al. 2003). Therefore, the electrochemical characteristics between platinum and monochloramine were investigated with CV to determine the appropriate applied potential for monochloramine measurement. Davies (1985) used an applied potential of +800 mV compared to SCE for the anodic reaction of monochloramine on platinum and proposed accumulation of platinum surface oxides that required cathodic pretreatment. A cyclic voltammogram is shown in Fig. 4.2 for a chlorine microelectrode in a solution containing 9.8 mg/L as Cl2 of monochloramine as the electroactive species in 50 mM phosphate buffer solution as
the supporting electrolyte. The potential was scanned positively, forward scan, from -1.0 V, then switched to negative at +1.0V for the reverse scan with three replications. In the absence of monochloramine, there was no current change in the range of applied potential, indicating no oxidation-reduction reaction in a buffer solution was observed during CV. In a solution containing 9.8 mg/L as Cl₂ of monochloramine, the anodic current rapidly increased at around +200 mV where monochloramine started oxidation, and it appears that the monochloramine oxidation here follows the following anodic reaction stoichiometry between platinum and monochloramine, which was proposed by Davies (1985).

\[
\begin{align*}
2 \text{NH}_2\text{Cl} & \rightarrow \text{N}_2 + 2 \text{Cl}^- + 4 \text{H}^+ + 2 e^- \quad (4.1) \\
2 \text{Pt} + 2 \text{OH}^- & \rightarrow 2 \text{PtOH} + 2 e^- \quad (4.2) \\
2 \text{PtOH} + 2 \text{NH}_2\text{Cl} & \rightarrow \text{N}_2\text{H}_4 + 2 \text{HCl} + \text{O}_2 + \text{Pt} \quad (4.3)
\end{align*}
\]

After this potential, a plateau was observed, followed by a cathodic current increase around -200 mV, which indicates monochloramine reduction, as seen in Eq. (4.4). Piela (2003) investigated the electrochemical behavior of chloramine with rotating platinum and gold electrodes and proposed the following monochloramine reduction:

\[
\text{NH}_2\text{Cl} + 2e^- + 2\text{H}_2\text{O} \rightarrow \text{NH}_4^+ + \text{Cl}^- + 2\text{OH}^- \quad (4.4)
\]

The magnitude between anodic peak current (nA) and cathodic peak current (nA) was increased when monochloramine concentration increased from 9.8 mg/L as Cl₂ to 40.8 mg/L as Cl₂. From this cyclic voltammogram, it was observed that the chlorine microelectrode responded to monochloramine and its concentration, and it was apparent that any positive potential in the plateau after +200 mV would be suitable as an applied potential. Therefore, in the plateau range, calibration experiments to determine an
appropriate applied potential were conducted manually and the signal at + 550 mV was found to increase proportionally to the monochloramine concentration, with a highly stable electrode response. At this potential of +550 mV, a newly fabricated microelectrode was calibrated over a broad monochloramine concentration range to investigate the microelectrode’s detection range. Fig. 4.3 shows the sensitivity of the finished chlorine microelectrode toward various monochloramine concentrations (0.1 to 30.0 mg Cl₂/L) at an applied potential of +550 mV (Ag/AgCl reference electrode), pH 8.0, 23°C. The response time was less than 30 seconds. Although the microelectrode’s response was not linear between 0 and 30 mg/L as Cl₂, two linear ranges above and below 0.6 mg/L as Cl₂ were observed. Creating two linear calibration curves for these two ranges allowed calibration for the entire range of interest. Both calibration curves showed a good linear response between current and monochloramine concentration with an R² of 0.99, and the lower range calibration curve showed a more sensitive response via its steeper slope, indicating that a more detailed monochloramine concentration measurement is possible at low concentrations range below 0.6 mg/L as Cl₂.

4.4.1.2. Lifetime and regeneration

The useful lifetime of the microelectrode was found to depend on the contact time between monochloramine and platinum. After approximately 6 months, the slope of the calibration curve had decreased by approximately 95%. The reason for the loss of activity appears due to the accumulation of a platinum oxide (PtOH) surface layer on the tip of the microelectrode, represented as follows (Vijh et al. 1977; Snell et al. 1982):

\[
P t + H_2O \rightarrow PtOH + H^+ + e^- \quad (4.5)
\]
Regeneration by pretreatment using electrochemical methods, including cathodic pretreatment at a negative potential (Davies 1985; Tsaousis 1985; Silver 1967), after losing the electrode response were found to have no effect on microelectrode reactivation. Therefore, pretreatment using both physical (beveling) and chemical (etching) methods were used for regeneration of the used microelectrodes. After re-beveling and re-etching, the microelectrode’s original characteristics were recovered and the tip diameter was increased, which resulted in a higher response due to the increased tip surface area. From the results, even though the bigger tip diameter provided more sensitivity for measurement, it is suggested that the microelectrode be rebuilt with a smaller tip when working with thin biofilms in order to minimize damage to the biofilm structure and allow for greater spatial resolution.

4.4.1.3. Tip size effect

Fig. 4.4 shows the effects of tip diameter on monochloramine measurement. Microelectrodes with greater tip diameters are more sensitive (i.e., the calibration curves have greater slopes) because the electrode current is proportional to the area of the exposed electrode (Fig. 4.4), according to Fick’s diffusion law (Beans et al. 1925). While a larger tip size would be more useful for measuring lower monochloramine concentrations due to increased sensitivity, a small tip diameter would give better spatial resolution in drinking water biofilms, which are typically thin. Therefore, it is recommended to balance between sensitivity needs and tip size for practical applications because microprofiling of biofilms requires a small tip size. In this study, the tip size of the microelectrode for monochloramine penetration was adjusted to 10-15 µm to balance
providing sufficient electrode response while preventing biofilm structural damage during microprofile measurements.

**4.4.1.4. Electrode surface analysis using SEM with EDAX**

The microelectrode tip surface was analyzed with EDAX to detect oxide layer buildup on the Pt tip and the results supported the existence of PtOH at the microelectrode tip surface. The oxygen content was 11.1% (wt) at the Pt tip for a newly fabricated chlorine microelectrode; after 15 hours monochloramine contact time, the oxygen percentage increased to 14.8% (wt). Because of the increasing percent oxygen, it appears that PtOH forms at the microelectrode tip over time (Eq. (4.2) and (4.5)), decreasing the microelectrode response to monochloramine. It was also expected that platinum oxidation would increase in the presence of DO and microelectrode response would change according to DO concentration based on the reaction of PtOH and monochloramine (Eq. (4.3)). This was experimentally verified in the current research by bubbling oxygen at different concentrations.

**4.4.2. Evaluation of microelectrode response to chlorine species**

Experiments were conducted to evaluate whether the microelectrode was specific for monochloramine or whether additional chlorine species (i.e. free chlorine or dichloramine) produced an electrode response. Several solutions were prepared at varying Cl₂:N ratios (pH 7.0 and 23°C) and allowed to age 1 hour. Fig. 4.5 shows the measured residual total chlorine and monochloramine concentrations and the resulting microelectrode response for each solution. The measured total chlorine and
monochloramine concentrations in Fig. 4.5 depict a typical breakpoint chlorination curve. Below a Cl₂:N ratio of 5:1, chlorine and ammonia combined to form predominant monochloramine (i.e. total chlorine equals monochloramine). As the Cl₂:N ratio increased beyond 5:1, chloramine is oxidized, resulting in decreased monochloramine and total chlorine concentrations until the breakpoint is reached where essentially all chloramine is oxidized. Cl₂:N ratios greater than required for breakpoint resulted in proportional increases in the free chlorine residual. Measured total chlorine or monochloramine concentrations in Fig. 4.5 followed the theoretical breakpoint chlorination scheme, and electrode response corresponded to the total chlorine values. This indicated that, in addition to monochloramine, the fabricated microelectrode also responded to free chlorine and possibly dichloramine.

To further resolve whether the electrode responded to dichloramine, chloramine solutions were prepared at both pH 4.0 and pH 8.0 at 4:1 Cl₂:N ratio, and electrode responses were compared. The chloramine species change at these different pH values. Dichloramine is known to be the predominant chloramine species at pH 4.0 (Kirmeyer et al. 2004), while at pH 8.0, monochloramine is the main species (Jafvert et al. 1992; Vikesland et al. 2001). It is also assumed that at 4:1 Cl₂:N ratio, most chlorine species would be combined into monochloramine or dichloramine and that free chlorine would be minimal. Therefore, the monochloramine or dichloramine concentration would be the same as the total chlorine concentration at pH 4.0 or 8.0. Fig. 4.6(a) shows that the microelectrode also responded to dichloramine at pH 4.0 but with less sensitivity than monochloramine.
To further confirm that the microelectrode measures free chlorine, chlorine solutions were prepared at two Cl₂:N ratios (4:1 and 12:1). Based on the previous breakpoint analysis and Fig. 4.6(b), monochloramine predominates at a 4:1 Cl₂:N and the microelectrode response increased with monochloramine or total chlorine concentration. However, at the 12:1 ratio, beyond the breakpoint where it is assumed that the total chlorine is all free chlorine, a more sensitive electrode response was observed proportional to total chlorine concentration (Fig. 4.6(b)); this response resulted from the residual free chlorine in the sample, and it appears that the electrode is actually more sensitive to free chlorine than monochloramine.

Experiments described in this section concluded that the fabricated microelectrode in this study is a total chlorine microelectrode that measures free chlorine, monochloramine, and dichloramine; this discovery impacts how microelectrode experiments should be conducted. Moreover, the electrode response for each chlorine species was not identical to a given concentration (i.e. from Fig. 4.6(a) and 4.6(b), 1 mg/L as Cl₂ for free chlorine, monochloramine, or dichloramine did not produce the same electrode response), which implies that the microelectrode responded not to one specific chlorine species but to total chlorine, free chlorine, and chloramine mixtures when multiple species were present. Therefore, experimental conditions for experiments concerned with monochloramine research should be performed at controlled conditions where most chlorine species present are monochloramine (i.e. at pH 8.0 and 4:1 Cl₂:N); experiments for monochloramine in this chapter were conducted under these conditions.
4.4.3. Environmental conditions that interfere: dissolved oxygen, pH, chlorine to nitrogen mass ratio, and media

4.4.3.1. Dissolved oxygen

The effect of DO on the electrode response was evaluated in the range of 0-21% DO (0-8.2 mg/L) by weight in a 50 mM, pH 8.0 PB. An increased DO concentration in the test solution increased the electrode response compared to the control sample where no monochloramine was present and thus affected subsequent calibration curves (Fig. 4.7). However, even at DO saturation a linear relationship still existed between the electrode response and monochloramine concentration. It appears that if the measurement is conducted at DO saturation, low monochloramine concentration measurements could be a problem because of the observed decrease in sensitivity of the data below 5 mg/L monochloramine. However, even if the bulk fluid is saturated with DO, as soon as the microelectrode enters into the biofilm, the DO concentration would likely drop with biofilm depth, and the electrode response to monochloramine would become more sensitive as the DO decreased. The linear electrode response indicates that it is possible to measure monochloramine biofilm penetration, even at varying DO concentrations. Because the microelectrode has different responses with varying DO within the biofilm, it is therefore required to simultaneously measure DO and monochloramine and to construct a 3-dimensional calibration curve with DO concentration, electrode response, and monochloramine concentration plotted as a surface. Fig. 4.8 shows an example of a 3-D calibration surface for monochloramine determination based on DO concentration and electrode response.
4.4.3.2. pH

To evaluate the effect of pH on electrode response during biofilm penetration, a monochloramine stock solution was prepared at pH 8 as previously described and buffer solutions at different pHs (6-9) were then dosed with the stock monochloramine solution. After dosing, the solutions were quickly measured so that the total chlorine present remained essentially all monochloramine. Fig. 4.9(a) shows the influence of pH on electrode performance. In the pH range of most drinking water experiments (pH 6-9), there was little change in the standard calibration curve; the microelectrode had a good linear response. In addition, pH variances between bulk solution and inside nitrifying biofilm are typically less than 1 pH unit (Vijh et al. 1977). Therefore, it is concluded that pH effects on monochloramine measurement within nitrifying biofilm would be minimal.

4.4.3.3. Chlorine to nitrogen mass ratio

The effect of Cl₂:N mass ratio was also evaluated. Various Cl₂:N ratios (3:1-5:1) were established by using a fixed chlorine concentration and changing the ammonia concentrations. Fig. 4.9(b) shows the effects of various Cl₂:N ratios on monochloramine measurements. At higher ammonia concentrations (i.e. lower ratios), the response was slightly increased (Fig. 4.9(b)). The monochloramine or total chlorine concentration is the same at pH 8.0, and this additional response was from free ammonia which originated from adding excess ammonia. However, the electrode response was reasonably close at low monochloramine concentrations, such that the free ammonia effect could be considered negligible in the Cl₂:N range of interest (3:1-5:1).
4.4.3.4. Media

Fig. 4.9(c) shows the media effect on microelectrode response. The results show a decrease of the calibration curve slope in 50 mM PB solution due to the higher phosphate concentrations than in the 5 mM PB solution. It seems that the response decrease occurred as a result of either the phosphate concentration or simply an ionic strength effect. This indicates that a high ionic strength may generate ion interference on the microelectrode performance. The linear relationship, however, still existed, even at 50 mM PB. In the 5 mM PB solution, in a dechlorinated drinking water created using a GAC column, and in a 5 mM carbonate solution designed to provide high alkalinity, the microelectrode performance was excellent with a good linear response and minimal differences. Therefore, it is expected that monochloramine measurements under normal drinking water conditions should present no problems.

4.4.4. Measurement of monochloramine penetration within a biofilm

Measurements of monochloramine penetration into a biofilm were conducted in a multi-species biofilm. To simplify this biofilm analysis, the experiments were conducted in deoxygenated solution (0 mg/L DO). The initial monochloramine concentration was 10.9 mg Cl₂/L in the bulk solution and was maintained during measurements; microprofiles were recorded at 25 min and 45 min after initiation of monochloramine addition in pH 8 PB. Fig. 4.10 shows the monochloramine microprofiles measured within the biofilm at each time. The microprofiles were successfully measured and showed a well-defined penetration profile with each contact time. Zero on the abscissa corresponds to the biofilm surface and bulk fluid interface, with a negative sign
indicating distance (µm) from the biofilm surface into the biofilm. The biofilm thickness was 1,200 µm. Monochloramine concentrations at the biofilm surface decreased by 73-77% compared to the bulk phase monochloramine concentration. After 25 minutes of treatment, monochloramine penetrated 300 µm into the biofilm. This increased to approximately 600 µm after 45 minutes of monochloramine treatment. The monochloramine concentration decreased with biofilm depth, indicative of characteristics for diffusion within the biofilm. Longer time periods would be required to evaluate the simultaneous effects of reaction and diffusion (de Beer et al. 1994). Overall, the chlorine microelectrode, with a small tip diameter (5 to 15 µm), was successfully used to measure monochloramine microprofiles with high spatial resolution (50 µm), preventing biofilm structure disruption during measurements. Future research conducted in our group will demonstrate the simultaneous diffusion and reaction with fully penetrated monochloramine microprofiles, conducted in oxygenated solution, and by applying the 3-D calibration curve with more representative drinking water biofilms.

**4.5. Conclusions**

Chlorine microelectrodes (tip size 5-15 µm) based on platinum were fabricated, fully characterized, and evaluated under various environmental conditions. These newly developed chlorine microelectrodes showed responses to free chlorine, monochloramine, and dichloramine, and as a result they should be used where only one chlorine species dominates the system. For the application of monochloramine measurements, it is recommended that experimental conditions be established where most of the chlorine species present are monochloramine (i.e. at pH 8.0 and 4:1 Cl₂:N). Under these
conditions, the microelectrode exhibited fast, repeatable response times and good signal stability for various monochloramine concentrations (0.1 to 30.0 mg Cl₂/L) when an applied potential of +550 mV was used. The microelectrode was shown to experience DO interference, and it is suggested that a 3-dimensional calibration curve with DO concentration, electrode response, and monochloramine concentration plotted as a surface for monochloramine measurement inside biofilm be constructed. Results obtained for representative drinking water conditions showed the applicability of this chlorine microelectrode for monochloramine measurement. Monochloramine penetration within a biofilm was successfully monitored, which will allow future experiments to evaluate biofilm disinfection efficacy. This research provides fundamental information to aid us in gaining a better understanding of the chlorine microelectrode for in-situ applications, and for measuring biofilm monochloramine microprofiles. Combining these profiles with other biofilm constituent profiles will allow evaluation of the microbial activity in the biofilm during the disinfection process, including nitrification in chloraminated drinking water distribution systems.
Figure 4.1 Chlorine microelectrode and experimental apparatus for microprofiling.

(a) Fabricated chlorine microelectrode

(b) Microelectrode setup in flow chamber for microprofiling
Figure 4.2  Cyclic voltammogram of monochloramine (deoxygenated 50 mM phosphate buffer solution, pH 8.0, 4:1 Cl₂:N, and 23°C). Scan initiated at -1.0V vs. Ag/AgCl in positive direction at 100 mV/s.

Figure 4.3  Microelectrode monochloramine detection range (deoxygenated 5 mM phosphate buffer solution, pH 8.0, 4:1 Cl₂:N ratio, and 23°C).
Figure 4.4 Tip diameter effect on microelectrode performance (deoxygenated 50 mM phosphate buffer solution, pH 8.0, 4:1 Cl₂:N ratio, and 23°C).
Figure 4.5 Residual total chlorine and monochloramine concentrations as a function of Cl₂:N mass ratio after 1 hour aging (5 mM phosphate buffer solution, pH 7.0, and 23°C). For each Cl₂:N ratio, residual total chlorine or monochloramine was measured by a colorimetric test kit (Total chlorine: Hach-8167, Monochloramine: Hach-10200).
Figure 4.6 Microelectrode response to monochloramine, dichloramine, and free chlorine. Residual total chlorine was measured by a colorimetric test kit (Total chlorine: Hach-8167).

(a) Electrode response to monochloramine (pH 8.0) and dichloramine (pH 4.0) (5 mM phosphate buffer solution, 4:1 Cl₂:N ratio, and 23°C).

(b) Electrode response to monochloramine (4:1 Cl₂:N ratio) and free chlorine (12:1 Cl₂:N ratio) (5 mM phosphate buffer solution, pH 8.0, and 23°C).
Figure 4.7  Dissolved oxygen effect on microelectrode performance (50 mM phosphate buffer solution, pH 8.0, 4:1 Cl₂:N ratio, and 23°C).
Figure 4.8  Example 3-D calibration surface for determination of monochloramine concentration using both DO concentration and microelectrode response (50 mM phosphate buffer solution, pH 8.0, 4:1 Cl₂:N ratio, and 23°C).
(a) pH effect on microelectrode performance (deoxygenated 50 mM phosphate buffer solution, 4:1 Cl₂:N ratio, and 23°C).

(b) Cl₂:N mass ratio effect on microelectrode performance (deoxygenated 50 mM phosphate buffer solution, pH 8.0, and 23°C).
(c) Media effect on microelectrode performance (deoxygenated media, pH 8.0, 4:1 Cl₂:N ratio, and 23°C).

Figure 4.9 pH, Cl₂:N mass ratio, and media effect on microelectrode response
Figure 4.10  Monochloramine concentration microprofile within biofilm recorded at 25 and 45 minutes after treatment with monochloramine (deoxygenated 50 mM phosphate buffer solution, pH 8.0, 4:1 Cl₂:N ratio, and 23°C)
Chapter 5. Investigation into Monochloramine Biofilm Penetration:
Penetration and its Effect on Biofilm Activity and Viability

5.1. Abstract

Chloramination has replaced free chlorine for secondary disinfection at many utilities due to disinfection by-product (DBP) regulations. However, there is a potential for nitrification that utilities must consider in their monitoring and maintenance practices when using chloramines. Nitrifying bacteria, which are responsible for nitrification in the distribution system, are well known to grow in clumps or aggregates by adhering to a solid surface (i.e. plumbing materials). Control of this biofilm is necessary to ensure water quality and protect public health. Many biofilm control strategies have been studied, and it was suggested that a less reactive biocide (i.e. monochloramine) that has a greater biofilm penetration than free chlorine be used, as it can result in better disinfection because of its persistence. However, viable counts have often been used to show disinfection efficacy in the biofilm, and no direct measurement of disinfectant within the biofilm has been conducted previously. In this study, monochloramine penetration and its effects on nitrifying biofilm activity and viability in chloraminated water systems were investigated and evaluated using microelectrodes and confocal laser scanning microscopy (CLSM). Two experiments concerning monochloramine disinfection were conducted with and without excess ammonia. Disinfectant penetration, biofilm activity, viability and recovery were compared under both conditions. Monochloramine and DO penetration within a nitrifying biofilm were successfully profiled, showing the reduction of aerobic activity via DO penetration following
application of monochloramine. Monochloramine penetrated fully into the nitrifying biofilm within 24 hours when fed at a 4:1 Cl₂:N ratio. However, the presence of excess ammonia prevented microbial deactivation. When monochloramine fully penetrated, bacteria were not all dead but some were simply inactive. It was concluded that monochloramine penetration did not necessarily equal a loss in viability from the Live/Dead results. Biofilm recovery occurred when disinfection stopped. Recovery was increased when excess ammonia was present during disinfection. This research provides more understanding of the relationship between lower reactive biocide (i.e. monochloramine) penetration and microbial activity during disinfection.

**Keywords:** Biofilm; confocal laser scanning microscopy (CLSM); microelectrode; microbial activities; monochloramine; nitrifying biofilm; penetration; recovery

### 5.2. Introduction

Many utilities have switched or are contemplating a switch from chlorine to chloramines as a secondary disinfectant to comply with the Disinfectants/Disinfection By-products Rule (D/DBPR) (USEPA 1998; USEPA 1979; USEPA 2005; Seidel et al. 2005). A recent survey reported that approximately 30% of U.S. drinking water plants use chloramines for disinfection (AWWA 2000; Betts 2002), and 12% of drinking water utilities are contemplating a switch to chloramines in the future (Seidel et al. 2005). Chloramines offer several benefits, including lower trihalomethanes (THM) and haloacetic acids (HAA), lower coliform regrowth in distribution pipes, and improved maintenance of disinfectant residual (Norton et al. 1997). Although less effective disinfectants than free chlorine, they appear to be more effective in controlling biofilm
microorganisms because they interact minimally with capsular polysaccharides. There is a report that hospitals using free chlorine as the residual disinfectant are 10 times more likely to experience outbreaks of Legionnaires’ disease, which is caused by Legionella bacteria which live in biofilm in natural and synthetic aquatic environments, than those using monochloramine (Kool et al., 2000). LeChevallier et al. (1990) suggested the use of free chlorine as a primary disinfectant in water distribution systems and to convert the residual to monochloramine, if biofilm control is the goal. Meanwhile, chloramines have some known drawbacks such as increasing nitrification and gasket material failures (Seidel et al. 2005). Chloramination may promote the growth of nitrifying bacteria (Wilczak et al. 1996) due to the ammonia which is released from chloramine decay during auto-decomposition, oxidation of natural organic matter (NOM), corrosion, catalysis at pipe surfaces and oxidation of nitrite under various conditions in the chloraminated water system (Kirmeyer et al, 2004), and may generate nitrification in the pipe systems by an attachment of nitrifiers to the pipe surface as shown in Figure 5.1. This attachment of nitrifiers to solid surfaces enhances their growth, and their presence in the biofilm material contributes to the survival of nitrifying bacteria (i.e. ammonia oxidizing bacteria (AOB) or nitrite oxidizing bacteria (NOB)) in the distribution system because of the excess essential nutrients and protection from the disinfectant (Wolfe et al. 1988; Wolfe et al. 1990). Camper et al. (1996) pointed out that a significant fraction of the bacteria grown was associated with the biofilms in distribution systems. The presence of this kind of biofilm in drinking water distribution systems contributes to the deterioration of water quality in the systems and results in public health concerns.
Many biofilm control strategies have been studied, and it was suggested that a lower reactive biocide (i.e. monochloramine) has a greater biofilm penetration and, therefore, can result in better disinfection because of its persistence (LeChevallier et al. 1990). However, viable counts were often used to show disinfection efficacy in the biofilm, and the relationship between disinfectant (i.e. lower reactive biocides) penetration and its effects on biofilm activity and viability were not investigated and evaluated.

In this study, microelectrodes were used as important experimental tools to measure directly monochloramine biofilm penetration and nitrifying microbial activities. Microbial viability upon the administration of chloramine disinfectant was investigated by observing confocal laser scanning microscopy (CLSM) with LIVE/DEAD® BacLight™. LIVE/DEAD® BacLight™ is a culture-independent membrane integrity based technique. A cell was considered viable or nonviable based on the ability of propidium iodide (PI) to penetrate its membrane and subsequent processing according to the method of LIVE/DEAD® BacLight™. The results from this study will provide answers for the following primary questions: 1) Does monochloramine fully penetrate into the biofilm? 2) How fast and how deep does monochloramine penetrate? 3) Are bacteria really dead or simply inactive when monochloramine fully penetrates? 4) If there is excess ammonia in the chloraminated water system, what is the response from the bacteria? 5) Do the bacteria which are deactivated recover? With these questions, the monochloramine biofilm penetration was investigated and will be discussed.

5.3. Materials and methods

5.3.1. Nitrifying biofilm growth and development
Nitrifying bacteria were inoculated from a distribution system experiencing nitrification and were then grown in an annular biofilm reactor (BioSurface Technologies Corp., Bozeman, MT, Model 1320 LJ) with approximately 200 mg N/L, 12 mg P/L, and 8 mg C/L over 4 months to simulate the conditions of nitrifying biofilm growth in a drinking water distribution system (Figure 5.2). The schematic diagram of the reactor operation and its operational performance results are shown in Figure 7.1 and Table 7.1. All biofilm samples tested in this experiment were taken from Reactor 3. Biofilm was grown under high flow velocity and shear. Therefore, this biofilm was evenly and well distributed with high density. Details on the annular biofilm reactor operation and results, and nitrifier inoculation and nitrifying biofilm development, will be described in more detail in Chapter 7 (sections 7.3.1 and 7.3.2).

5.3.2. Microelectrode preparation

Chlorine type microelectrodes for measuring monochloramine, and dissolved oxygen (DO), ammonium (NH$_4^+$), nitrate (NO$_3^-$), and pH microelectrodes were fabricated in the laboratory and used to measure the constituent concentration microprofiles within biofilms. The chlorine microelectrode used for measurement of monochloramine followed the design of a previously described chlorine microelectrode (de Beer et al, 1994). A platinum wire (0.127 mm diameter, 99.99% purity, Aldrich Chemical Co.) was etched in a 6 M potassium cyanide (KCN) solution. The etched wire was inserted into a pulled glass micropipette (O.D.: 1.5 mm, I.D.: 0.75 mm, 15 cm length, World Precision Instruments) and sealed with heat as described previously (Bishop et al, 1999; Yu, 2000; Wang, 2005). The resulting tip diameter of the fabricated chlorine microelectrode was
less than 15 µm. The fabricated chlorine microelectrode was calibrated in different monochloramine concentrations (0.5 to 16.1 mg Cl₂/L) at 550 mV of applied potential with an Ag/AgCl reference electrode at pH 8.0 and 23°C. Details of the sensing mechanism, fabrication procedures and calibration of chlorine microelectrodes for monochloramine measurement were described previously in Chapter 4. Ion-selective microelectrodes (pH, NO₃⁻, NH₄⁺) and DO microelectrodes will be described in Chapter 7.

5.3.3. Monochloramine disinfection experiment and microprofiling

Two monochloramine disinfection experiments were conducted to investigate monochloramine penetration within nitrifying biofilm and its effect on the biofilm activity and viability. One experiment involved monochloramine disinfection in the simulated chloraminated water system. 4.4 mg Cl₂/L monochloramine (4:1 Cl₂:N) was prepared in 5 mM pH 8.0 buffer solution (1510-4L, Aqua Solutions, Inc., Deer Park, TX) by adding a calculated amount of a sodium hypochlorite solution (5% available chlorine, No.:7495.5-32, Ricca Chemical, Fisher Scientific, Pittsburgh, PA) and ammonia stock solution (100 mg N/L). This monochloramine solution was stored at 23°C for several hours before the experiments. In the beginning of the experiment, air was saturated to maintain 8.3 mg/L of DO concentration in the monochloramine feed solution. Preliminary experiments showed the air bubbling did not affect the monochloramine concentration within the disinfection time (i.e. 24 hours). Then, a polycarbonate slide that had a well developed nitrifying biofilm growing on it was taken from reactor 3. Biofilm tested in this experiment was carefully selected to be a biofilm which is well developed and distributed with high density, in the range of 250 – 350 µm biofilm
thickness. This slide was cut into five pieces from its original 15 cm long slide. These pieces were used for microprofiling and CLSM imaging with fluorescent stain. Four of these five pieces were inserted into the flow chamber for microprofiling, the monochloramine disinfection experiment, and the preparation of the samples for CLSM analyses, as shown in Figure 5.3(a). Four CLSM images were taken: 1) intact sample, which represents live cells; 2) sample at a time when monochloramine penetrated to about half of the initial concentration into the bottom of the biofilm; 3) sample with full monochloramine penetration; and 4) sample after several days of feeding ammonia for the recovery after disinfection. The time for the CLSM image of sample 4 was determined based on the results of the DO microprofiles which were measured with time interval after the monochloramine disinfection. LIVE/DEAD® BacLight™ (L7012, Molecular Probes, Inc, Eugene, OR) was used to stain live and dead cells in biofilm before and after monochloramine disinfection, and disinfection results were determined with Confocal Laser Scanning Microscopy (Zeiss LSM 510 CLSM) (see section 7.3.5. in Chapter 7). DO, pH, ammonium, and nitrate were measured in one piece of biofilm, indicating the intact nitrifying microbial activity at time 0. The performance of microprofile measurements, including media used for flowing fluid, will be described in Chapter 7 (section 7.3.3.5) in detail. Then, DO and monochloramine microelectrode was positioned at 1,000μm above the biofilm-water interface, and monochloramine was injected by flow rate control valve with a flow rate of 15 mL/min. Measurements were recorded at 50 – 100 μm intervals into the biofilm. Figure 5.3(b) shows the monitoring of the chlorine microelectrode approaching to the biofilm surface. When complete monochloramine penetration was measured with time, the feed of monochloramine was
stopped, and DO, pH, ammonium, and nitrate were measured to monitor the nitrifying microbial activities. Then, ammonia (4.2 mg NH$_3$-N/L) was fed to investigate the biofilm activity recovery and DO microprofiles were measured with time. When DO no longer changed and stable DO microprofiles were measured with time, DO, pH, ammonium, and nitrate were measured again to investigate the recovery.

For the second experiment, excess ammonia (4.2 mg NH$_3$-N/L) was fed simultaneously with monochloramine. 5.3 mg Cl$_2$/L monochloramine (4:1 Cl$_2$:N) was prepared in 5 mM pH 8.0 buffer solution (1510-4L, Aqua Solutions, Inc., Deer Park, TX) by adding a calculated amount of a sodium hypochlorite solution (5% available chlorine, No.:7495.5-32, Ricca Chemical, Fisher Scientific, Pittsburgh, PA) and ammonia stock solution (100 mg N/L). The other procedures for CLSM analyses, DO and monochloramine microprofile measurements and recovery were the same, except for pH, ammonium, and nitrate microprofiling.

One control experiment was performed to assess the influence of the substratum on electrode response prior to these two experiments. The monochloramine profile in the flowing fluid adjacent to a clean substratum (i.e. polycarbonates slide with no biofilm of any kind) was flat (data not shown), indicating that the substratum used for growing nitrifying biofilm in the annular reactors did not disturb monochloramine measurement by the microelectrode.

5.3.4. CLSM observation

The details will be described in section 7.3.5. in Chapter 7.
5.4. Results and discussion

5.4.1. Monochloramine disinfection

5.4.1.1. Activity prior to disinfection (feeding 4.2 mg NH₃-N/L)

Before disinfection, the initial nitrifying microbial activities were investigated using microelectrodes. Figure 5.4(a) shows DO, ammonium, pH, and nitrate microprofiles in an intact nitrifying biofilm sample before the monochloramine disinfection experiment. All available DO was utilized near the surface of the biofilm. Ammonium decreased from 4.2 mg/L to 2.6 mg/L at 300 μm inside biofilm, indicating the ammonium diffusion and consumption by microbial activity. pH also decreased because of alkalinity consumption by nitrification, and nitrate increased up to 1.5 mg/L inside the biofilm. These concentration microprofiles describe well defined nitrifying biofilm microbial activity. A CLSM image of intact biofilm confirmed that the cells were alive (i.e. bacteria with intact cell membranes) in the biofilm at time 0 (i.e. initial condition before monochloramine disinfection) (Fig. 5.7(a)).

5.4.1.2. Monochloramine penetration and biofilm deactivation

4.4 mg Cl₂/L monochloramine (4:1 Cl₂:N) was injected and its penetration into the biofilm was measured with time. Figure 5.5 shows the monochloramine penetration microprofiles during the monochloramine disinfection (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 4.4 mg Cl₂/L monochloramine). At first, monochloramine concentration decreased with biofilm depth, indicating the typical characteristics for a chemical that is undergoing reaction and diffusion simultaneously. Monochloramine penetrated into the biofilm over time. Monochloramine penetrated to 50% of the bulk
concentration at the biofilm surface after 1 hr and to 50% of the bulk monochloramine concentration at the bottom of the biofilm after 2 hrs. In about 24 hours, monochloramine fully penetrated into the biofilm, and the diffusion boundary layer disappeared under this condition. It was concluded that the monochloramine diffusion rate was higher than the reaction rate, so monochloramine continued to penetrate into the biofilm. Figure 5.7(b) shows the CLSM image which was taken after 4 hours when monochloramine penetrated to about half of the initial concentration. The biofilm viability decreased from the initial state. However, mostly cells in the biofilm were observed to have intact membranes, even though some amount of monochloramine already existed inside the biofilm. When full monochloramine penetration was achieved after 24 hour monochloramine disinfection, microbial activity changes were investigated (Fig. 5.4(b)). DO also fully penetrated into the bottom of the biofilm, indicating apparent cessation of aerobic activity within the nitrifying biofilm upon the administration of chloramine disinfectant. pH, ammonium, and nitrate microprofiles were not changed, both inside and outside the biofilm, indicating that the nitrifying microbial activities were totally stopped due to the disinfectant addition. Alkalinity was not consumed, ammonium was not oxidized and nitrate was not produced under the fully penetrated monochloramine condition. The diffusion boundary layer for all constituents also disappeared, and it seemed that there was no barrier preventing ammonium and oxygen from being transported. All evidence from these microprofiles confirmed the microbial deactivation. However, microbial viability could not be ascertained from just the microprofiles. From the CLSM image taken after 24 hours monochloramine disinfection (Fig. 5.7(c)), it was confirmed that cells in the biofilm were not killed, but were
inactivated. Biofilm viability was seen to decrease with time. However, some cells with intact membranes on the surface could still be observed, even under full monochloramine penetration.

5.4.1.3. Biofilm activity recovery after disinfection

When monochloramine and DO full penetration into the biofilm was observed from the microprofiling, we investigated the possibility of microbial activity recovery achieved by feeding ammonia under the proper environmental conditions for nitrifier (i.e. pH 8.0, 23°C, 8.3 mg/L DO, and 4.2 mg N/L ammonia). Figure 5.6 shows the DO microprofile changes with time when fed ammonia. Biofilm aerobic microbial activity increased over time and 40% of DO consumption was recovered after 163 hrs (6.8 days). It appears that the nitrifiers recovered their activity over time in the absence of chloramines, even though they once had been exposed to relatively high concentrations of monochloramine. One example of a nitrification episode in a South Australian distribution system supports the microbial deactivation and subsequent recovery following chloramination (AWWA 2006; Cunliffe 1991). When residual total chlorine was maintained at an average 2 mg/L as Cl₂, nitrification was controlled the nitrifying bacteria count (per mL) decreased. However, as residual total chlorine decreased, nitrification began again, with an increase of nitrate concentration and oxidized nitrogen in the distribution system. Griebe et al. (1994) observed the regrowth after treatments with monochloramine. Figure 5.7(d) shows the CLSM image after 163 hrs (6.8 days) of ammonia feeding after full monochloramine penetration. Cells without damaged membranes, green colored ones,
were observed at the surface of the biofilm. This result indicates that the bacteria were simply inactive during the chloramine disinfection.

5.4.2. Monochloramine disinfection with excess ammonia

5.4.2.1. Monochloramine penetration and biofilm deactivation

In the second experiment, excess ammonia (4.2 mg NH$_3$-N/L) was added simultaneously during disinfection. Since nitrifiers, including AOB, thrive on ammonia, it was expected that aerobic microbial activity would remain under this condition. Figure 5.8 shows the progression of monochloramine penetration and DO microprofiles with time during monochloramine disinfection with excess ammonia. Over the first few hours, monochloramine penetration followed the same pattern as in the former experiment. As monochloramine penetrated into the biofilm, the aerobic activity of the biofilm also decreased and the DO began to penetrate the biofilm. However, after 4 hours, the monochloramine penetration and DO consumption stabilized. In 24 hours, only 54% of the monochloramine penetrated, and fully penetration was not observed (Fig. 5.8(a)). The diffusion boundary layer for monochloramine remained at around 250 $\mu$m thick. The diffusion boundary layer for DO also decreased with time, but 30% of the DO was still consumed inside the biofilm by microbial activity (Fig. 5.8 (b)). This result pointed out that some of nitrifying bacteria in the biofilm were still active in the presence of excess ammonia in the chloraminated water, indicating the penetration limitation of the lower active biocide due to microbial activity. Figure 5.10 shows the progression of the biofilm during monochloramine disinfection with excess ammonia by observing CLSM images at the same time intervals during microprofiling. Biofilm viability continued to decrease with monochloramine contact time. One interesting result was that, even though
monochloramine penetration depth and DO consumption rate after 4 hours and after 24 hours monochloramine disinfection were similar (Fig. 5.8), CLSM images taken at corresponding times show differences in biofilm viability, as shown in Fig. 5.10 (b) and (c). Dead cells with damaged membranes increased with time. However, live cells with intact membranes at the biofilm surface were still observed, even at 24 hours.

5.4.2.2. Activity recovery comparison with DO microprofiles

Figure 5.9 shows the DO microprofiles after monochloramine disinfection with excess ammonia. The conditions for monitoring of microbial activity recovery were the same as in the former experiment (i.e. pH 8.0, 23°C, 8.3 mg/L DO, and 4.2 mg N/L ammonia). Two DO microprofiles measured with biofilms which experienced different monochloramine and DO penetration were compared. In the biofilm where monochloramine disinfection was conducted with excess ammonia, faster recovery was observed. Based on DO consumption at 250 \( \mu \text{m} \) inside the biofilm, 40% of the DO was consumed at 91 hours (3.8 days) due to microbial recovery in the biofilm in the first disinfection experiment (Fig. 5.6), while 70% of the DO was consumed at 94 hours (3.9 days) in the biofilm during the second disinfection experiment (Fig. 5.9). Comparison of CLSM images provided evidence of more live cells at the biofilm surface following recovery (Fig. 5.7(d) and Fig. 5.10(d)).

5.5. Conclusions

Monochloramine and DO penetration within a nitrifying biofilm were successfully profiled, showing the reduction of aerobic activity via DO penetration following
application of monochloramine. Monochloramine penetrated fully into the nitrifying biofilm within 24 hours when fed at a 4:1 Cl₂:N ratio, but not in the presence of excess ammonia, which prevented microbial deactivation. When monochloramine fully penetrated, bacteria were not all killed, but were simply inactive. It was concluded that monochloramine penetration did not necessarily equal a loss in viability, based on the Live/Dead results. Biofilm recovery was possible when disinfection stopped and when there were sufficient nutrients, including ammonia. This research provides more understanding of relationships between lower reactive biocide (i.e. monochloramine) penetration and microbial activity during disinfection.
Figure 5.1 Nitrification occurrence in the chloraminated drinking water distribution system biofilm.

Figure 5.2 Annular biofilm reactors for nitrifying biofilm development (The last reactor on the right is reactor 3 where biofilm samples for this study were grown).
Figure 5.3  Performance of microprofile measurements.
(a) Intact biofilm (before disinfection)

(b) 24 hour monochloramine disinfection (fully penetration)
(c) 163hrs (6.8days) of ammonia feeding after the disinfection

Figure 5.4 Microprofiles of dissolved oxygen, ammonium, pH and nitrate in the nitrifying biofilm before and after the monochloramine disinfection (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 4.4 mg Cl₂/L monochloramine)
Figure 5.5 Monochloramine penetration microprofile during monochloramine disinfection (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 4.4 mg Cl₂/L monochloramine)

Figure 5.6 DO microprofiles after monochloramine disinfection
(a) Intact biofilm (before disinfection) (Z stack: 180 μm)

(b) 4 hour monochloramine disinfection (Z stack: 95 μm)
Figure 5.7 Progression of CLSM images of the biofilm during monochloramine disinfection (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 4.4 mg Cl₂/L monochloramine) and its recovery after disinfection. Green and red coloring indicates live and dead cells, respectively.
Figure 5.8  Microprofiles of monochloramine and DO during monochloramine disinfection with excess ammonia (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, 5.3 mg Cl₂/L monochloramine, and 4.2 mg NH₃-N/L ammonia)
Figure 5.9  DO microprofiles after monochloramine disinfection with excess ammonia
(a) Intact biofilm (before disinfection) (Z stack: 122.1 µm)

(b) 4 hour monochloramine disinfection (Z stack: 128.9 µm)
Figure 5.10  Progression of CLSM images of the biofilm during monochloramine disinfection with excess ammonia (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, 5.3 mg Cl₂/L monochloramine, and 4.2 mg NH₃-N/L ammonia) and its recovery after disinfection.  Green and red coloring indicates live and dead cells, respectively.
Chapter 6. Comparison of Disinfectant Penetration and Reaction-Diffusion Interaction between Monochloramine and Chlorine in Water Distribution Systems Biofilms

6.1. Abstract

The presence of biofilm in drinking water systems is undesirable and may result in degradation of water quality and subsequent non-compliance with existing regulations; therefore, effective biofilm control may be necessary to maintain public health safety. Biofilms have significant resistance compared with suspended microorganisms during disinfection, and a lower reactive biocide (i.e. chloramines) has been suggested to provide better biofilm penetration. However, the bases for biofilm resistance to chlorine and monochloramine biofilm penetration remain incompletely understood, and no direct measurements between monochloramine and chlorine have been conducted to compare the penetration ability into the biofilm and its correlation to microbial viability in simulated drinking water distribution system biofilms. In this study, disinfectant penetration of monochloramine and chlorine were compared and the impact of disinfectant penetration on viability of biofilms in environments and the diffusion-reaction interaction between monochloramine and chlorine were investigated and evaluated for biofilm control. Monochloramine showed greater penetration compared to chlorine, indicating the apparent cessation of aerobic activity within the nitrifying biofilm upon the administration of chloramine disinfectant. Monochloramine penetrated into the biofilm surface layer 49 times faster than chlorine within the nitrifying biofilm and 39
times faster in the multi-species biofilm than did chlorine. On the other hand, free chlorine seems to be consumed by oxidizing the organic matter in path of the biofilm, then diffuse further, indicating very slow chlorine penetration. However, CLSM image of membrane compromised cells in biofilm after chlorination confirmed that free chlorine is much stronger than monochloramine in disinfection. Comparison of DO microprofiles, which were measured during the chlorine disinfection and monochloramine disinfection respectively, showed that the cessation of aerobic microbial activity was more complete under chloramination. The time required to attain 50% of the monochloramine biofilm penetration near the substratum was average less than 2 hours in this research. On the other hand, chlorine showed only a maximum of 9% penetration during the times when experiments were conducted. The results in this study support the hypotheses that monochloramine penetrates more quickly and further into the biofilm than chlorine and monochloramine and free chlorine act differently at surfaces of biofilm composed of various bacteria, and provides useful information for use of disinfectants for biofilm control.

**Keywords:** Biofilm; chlorine; CLSM; diffusion-reaction interaction; distribution system; microelectrode; monochloramine; penetration

### 6.2. Introduction

The presence of biofilm in drinking water systems such as distribution systems, reservoirs, and treatment facilities generates water quality issues (e.g. disinfectant depletion, coliforms occurrences) and compliance issues (e.g. violation of the surface water treatment rule, total coliform rule, or lead and copper rule), and effective biofilm
control may be necessary to maintain the public health safety. It is well known that nitrification can be generated due to the detrimental development of nitrifying biofilms attached to pipe surfaces, which consist of ammonia oxidizing bacteria (AOB) and/or nitrite oxidizing bacteria (NOB) in the distribution system. These are caused by ammonia which is released from chloramine decay in the chloraminated drinking water (Wilczak et al. 1996; Kirmeyer et al. 2004; Wolfe et al. 1988; Wolfe et al. 1990). Nitrification within drinking water distribution systems reduces water quality, causes difficulties with maintaining adequate disinfectant residual, and poses public health concerns including exposure to nitrite, nitrate, and opportunistic pathogenic microorganisms (AWWA, 2006).

Biofilm control is often accomplished with antimicrobials (i.e. strong oxidizing agents and disinfectants) such as chlorine. Chlorine and monochloramine are commonly used as secondary disinfectants, with many utilities currently switching from chlorine to monochloramine to comply with disinfection by-product regulations. (USEPA 1998; USEPA 1979; USEPA 2005; Seidel et al. 2005; AWWA 2000; Betts 2002). There are many reports which support the observed resistance of biofilm micro-organisms to disinfection when compared with dispersed cells of the same organisms (Stewart et al. 2001; LeChevallier et al. 1988a; LeChevallier et al. 1988b; Sharma et al. 1987). van der Wende et al. (1989) proposed that the biofilm environment protects cells against chlorine by diffusional resistance and neutralization of the chlorine. Stewart (1995) used the reaction-diffusion model to explain this resistance as transport limitation and limited penetration of antimicrobial agents into the biofilm. Meanwhile, it was suggested that a lower reactive biocide has greater biofilm penetration and therefore could result in better
disinfection because of its persistence (LeChevallier et al. 1990). Monochloramine is believed to penetrate biofilms better compared to chlorine, and their long-term stability in drinking water distribution systems and tendency to form low levels of regulated disinfectant by-products (DBPs) provide a positive effect on disinfection performance (Seidel et al. 2005). Evidence that chlorine penetration into biofilms can be profoundly retarded has been presented recently (de Beer et al. 1994; Chen et al. 1996; Xu et al. 1996), and LeChevallier (1988) suggested that monochloramine and free chlorine act differently at surfaces of biofilm composed from various bacteria. However, the basis for biofilm resistance to chlorine remains incompletely understood (Stewart et al. 2001), and no direct measurements between monochloramine and chlorine have been conducted to compare the penetration abilities into biofilm and their correlation to the microbial viability in simulated drinking water distribution system biofilms.

In this study, the disinfectant penetration between chlorine and monochloramine was compared, the impact of disinfectant penetration on viability of biofilms in drinking water distribution system biofilms and the diffusion-reaction interaction between monochloramine and chlorine were investigated and evaluated for biofilm control. Chlorine microelectrodes, manufactured using platinum wire, were fabricated and used to measure chlorine and monochloramine microprofiles within biofilms. Direct concentration measurements in biofilms were conducted with high spatial resolution using chlorine microelectrodes. Nitrifying biofilms, which were thin (250 - 350 μm), dense and well-distributed on the surface of a simulated chloraminated drinking water distribution system, were used to determine disinfectant penetrations into a microbial biofilm during disinfection with chlorine and monochloramine. Relatively thick biofilms
(830 - 1,300 μm thickness) were also tested to compare the penetration of these two disinfectants with higher concentrations (800 mg Cl₂/L). Microbial viability resulting from the administration of chlorine and chloramine disinfectant was investigated by using confocal laser scanning microscopy (CLSM) with LIVE/DEAD® BacLight™. LIVE/DEAD® BacLight™ is a culture-independent method, which relies on cell membrane integrity as a viable measure. Propidium iodide (PI) penetrates only bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present in bacteria. Therefore, viability was expressed based on the ability of propidium iodide (PI) to penetrate cell membrane and the subsequent processing according to the method of LIVE/DEAD® BacLight™.

6.3. Materials and methods

6.3.1. Biofilm growth and development

Two kinds of biofilm were used for biofilm disinfection experiments: nitrifying biofilms and multi-species biofilms. Nitrifying biofilms were grown on polycarbonate slides in an annular biofilm reactor (BioSurface Technologies Corp., Bozeman, MT, Model 1320 LJ) fed with approximately 200 mg N/L, 12 mg P/L, and 8 mg C/L over 4 months to simulate the conditions of nitrifying biofilm growth in a drinking water distribution system (Fig. 5.2 and Fig. 7.1). All nitrifying biofilm samples tested in this experiment were taken from Reactor 3. Biofilm was evenly and well distributed with high density because of the operating conditions of high flow velocity and shear. Details on the annular biofilm reactor operation and results, and nitrifier inoculation and nitrifying biofilm development, will be described in more detail in Chapter 7 (sections
7.3.1 and 7.3.2). Multi-species artificial biofilms were formed in a laboratory water system (see section 3.3.6) using submerged glass slides (12-544-5CY, Fisher Scientific) as support surfaces under a relatively high substrate concentration (200 mg C/L and 20 mg N/L), thus representing a thick and dense biofilm, was also used for the biofilm disinfection experiments.

6.3.2. Microelectrode preparation

The monochloramine and chlorine microelectrodes, produced using platinum wire, were fabricated to measure the concentration microprofiles within biofilms at different applied potentials (i.e. monochloramine: +550mV, chlorine: +200mV) (Lee et al. 2009b). Details of the sensing mechanism, fabrication procedures and calibration of chlorine microelectrodes for monochloramine measurement were described previously in Chapter 4. The dissolved oxygen (DO) microelectrodes were used to investigate the aerobic microbial activity changes during chlorine and monochloramine experiments. DO microelectrodes are polarographic recessed cathode gold electrodes with small tip diameters (~5 μm). DO microelectrodes will be described in Chapter 7. A set of DO microelectrode/chlorine microelectrode or DO microelectrode/monochloramine microelectrode was prepared in one holder which was controlled by a three-dimensional micromanipulator (World Precision Instruments, M3301) during each disinfectant penetration experiment.
6.3.3. Disinfection experiments and microprofiling

6.3.3.1. Experiment with nitrifying biofilm

Nitrifying biofilms grown for 6 months (250 – 350 μm thickness) were subjected to two kinds of in situ disinfectant treatments by simply switching solutions. Two experiments were conducted: a chlorine penetration experiment and a monochloramine penetration experiment. The chlorine penetration experiment consisted of two phases: Phase 1 (disinfection with chlorine of 2.6 mg/L as Cl₂ during 24 hours) and Phase 2 (continuous chlorine disinfection with higher concentration of 9.6 mg/L as Cl₂ after Phase 1). The monochloramine penetration experiment consisted of two phases: Phase 1 (disinfection with monochloramine of 2.7 mg/L as Cl₂ during 24 hours) and Phase 2 (changed to chlorine disinfection of 2.8 mg/L as Cl₂ after Phase 1). Biofilm profile times for Phase 1 (i.e. 24hrs) in each experiment were set based on the results in the previous monochloramine penetration experiments described in Chapter 5, where complete monochloramine penetration was measured within 24 hrs. 2.7 mg Cl₂/L monochloramine (4:1 Cl₂:N) was prepared in 5 mM pH 8.0 buffer solution (1510-4L, Aqua Solutions, Inc., Deer Park, TX) by adding a calculated amount of a sodium hypochlorite stock solution (5% available chlorine, No.:7495.5-32, Ricca Chemical, Fisher Scientific, Pittsburgh, PA) and ammonia stock solution (100 mg N/L). This monochloramine solution was stored at 23°C for several hours before the experiments. In the beginning of the experiment, air was saturated to maintain 8.3 mg/L of DO concentration in the monochloramine feed solution. Preliminary experiments showed the air bubbling did not affect the monochloramine concentration within the disinfection time (i.e. 24 hours).
Several chlorine solutions (i.e. 2.6, 9.6, and 2.8 mg Cl₂/L) were prepared in 5 mM pH 8.0 buffer solution by adding a calculated amount of a sodium hypochlorite solution.

A biofilm sample from a removable slide was cut into five pieces from the original 15 cm long slide. These pieces were used for microprofiling and CLSM imaging with fluorescent stain. Four of these five pieces were inserted into the flow chamber for DO and disinfectant penetration microprofiling in the each disinfection experiment and the preparation of samples for CLSM analyses. Three CLSM images were taken: 1) intact sample, which represents live cells; 2) sample at a time when disinfectant penetrated to about half of the initial concentration into the bottom of the biofilm; 3) sample after Phase 1; and 4) sample after Phase 2. CLSM images with different contact times and disinfectant concentrations were also taken as necessary. LIVE/DEAD® BacLight™ (L7012, Molecular Probes, Inc, Eugene, OR) was used to stain live (i.e. bacteria with intact membranes) and dead cells (i.e. bacteria with damaged cell membranes) in biofilm before and after disinfection, and disinfection results were determined with Confocal Laser Scanning Microscopy (Zeiss LSM 510 CLSM) (see section 7.3.5. in Chapter 7).

DO and monochloramine microelectrodes were positioned at 1,000 µm above the biofilm-water interface, and monochloramine was injected into the flow chamber by a flow rate control valve with a flow rate of 15 mL/min (Fig. 5.3. and Fig. 7.2). Measurements were recorded at 50 – 100 µm intervals into the biofilm. Chlorine and monochloramine concentrations in the bulk solution were measured using colorimetric test kits (Total chlorine: Hach-8167; Monochloramine: Hach-10200) and a DR/2010 spectrophotometer (Hach Co.).
6.3.3.2. Experiment with multi-species biofilm

Multi-species biofilms grown over 3 months (830 - 1,300 μm thickness) were also subjected to two kinds of disinfectant treatments with relatively high concentrations of disinfectants in situ by simply switching solutions. Two experiments were applied; a chlorine penetration experiment and a monochloramine penetration experiment. 800 mg Cl₂/L monochloramine (4:1 Cl₂:N) was prepared in 5 mM pH 8.0 buffer solution by adding a calculated amount of a sodium hypochlorite stock solution and ammonia stock solution. 800 mg Cl₂/L chlorine was prepared in 5 mM pH 7.0 buffer solution by adding a calculated amount of a sodium hypochlorite stock solution. Microprofile measurements were carried out in a 5.0 mM buffer solution at pH 7.0 for chlorine and pH 8.3 for monochloramine at 23 °C in a flow chamber with 15 mL/min of flow rate, as shown in Figure 6.1. Nitrogen was bubbled into the feed solution in this experiment to purge oxygen and reduce the aerobic microbial activity. Microprofile measurements were conducted with the same procedures described in section 2.3.1

6.3.4. CLSM observation

The details will be described in section 7.3.5. in Chapter 7.

6.4. Results and discussion

6.4.1. Chlorine penetration

Figure 6.2 shows the chlorine and DO microprofiles achieved during chlorine disinfection (Phase 1: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.6 mg Cl₂/L chlorine). The chlorine penetration microprofile (Fig. 6.2.(a)) showed that chlorine
reacted rapidly with organic constituents in the biofilm, especially in surface layers of the biofilm, and its penetration was thereby retarded during 20 hours of chlorine disinfection. It seemed that the chlorine was reactively neutralized near the biofilm surface, and its ability to penetration the biofilm was severely compromised at this low concentration. The chlorine concentration at the biofilm surface was around 17.6% - 25% of the bulk concentration during the chlorine disinfection over 24 hours. The results describes a reaction-diffusion interaction in the biofilm leading to poor antimicrobial penetration, as was shown in previous research (Stewart et al. 2001; Stewart et al 2000; Anderl et al. 2000). Chlorine may not reach the cells due to diffusional resistance of the biofilm matrix (Costerton et al. 1987) or neutralization of the chlorine inside the biofilm matrix (Nicholos et al. 1989; van der Wende et al. 1989). One experiment on chlorine penetration into artificial biofilms (526 \mu m thick film containing 14,400 mg of \textit{Pseudomonas aeruginosa} cell mass/L) of entrapped in agarose gel slabs by Xiao et al. (1995) indicated that after 3 hrs of treatment with a flowing chlorine solution with 18.6 mg/L as \text{Cl}_2, the concentration of chlorine at the substratum only rose to 10% of the bulk solution value.

Figure 6.3 shows a progression of CLSM images of the biofilm during chlorine disinfection (Phase 1). CLSM images could only be obtained to about half way into the biofilm and Z stack (\mu m); each picture indicates the CLSM observation depth in which Z sectioning was performed. Fig 6.3 (a) shows much green color, which indicates the cells membranes in the biofilm were intact. As contact time increased, the viability decreased, and after 24 hours the color of the surface of the biofilm turned red, indicating all bacteria were damaged in their cell membranes. This result showed that free chlorine is much
stronger than monochloramine in disinfection. However, DO microprofiles (Fig. 6.2(b)) indicated that 29% DO at the 250 μm depth inside the biofilm was still utilized even after 20 hours chlorine disinfection, probably due to the chlorine limited penetration (Fig. 6.2(a)). Complete observation of the biofilm matrix with depth was not possible due to the limitations of dye penetration and CLSM observation depth.

If the diffusion-limited reaction prevents chlorine penetration due to the barrier at the surface of the biofilm at low concentration, it would be predicted that higher concentrations may increase the diffusion-reaction rate. In Phase 2, after 24 hours chlorine disinfection with a low concentration (i.e. 2.62 mg Cl₂/L chlorine), the chlorine concentration was increased to 9.6 mg Cl₂/L chlorine. Figure 6.4 shows the chlorine and DO both penetrated the biofilm with time after increasing the chlorine concentration. With the chlorine concentration in Phase 2, the chlorine concentration gradually decreased in the boundary layer (Fig. 6.4(a)).

6.4.2. Monochloramine penetration

The effects of simultaneous monochloramine and dissolved oxygen biofilm penetration are shown in Figure 6.5. At time 0 (i.e. prior to monochloramine addition), all available DO was utilized near the surface of the biofilm. Over the first few hours, as monochloramine penetrated into the biofilm, the aerobic activity of the biofilm decreased due to reactions with the monochloramine, and the DO began to penetrate the biofilm as well. Both diffusion boundary layers for DO and monochloramine were decreased and, in about 24 hours, monochloramine and DO both fully penetrated the biofilm without any boundary layer for diffusion. Together these two figures show the apparent cessation of
aerobic activity within the nitrifying biofilm upon the administration of chloramine disinfectant. Monochloramine (Fig. 6.5(a)) showed greater biofilm penetration compared to chlorine (Fig. 6.2(a)), and this result confirmed the hypothesis that monochloramine penetrates more quickly and further into the biofilm than chlorine. The monochloramine concentration change rate at the surface of the biofilm tested averaged 0.012 mg Cl$_2$/L·min (4.5 × 10$^{-4}$ - 2.5 × 10$^{-2}$ mg Cl$_2$/L·min) (Fig. 6.5(a)) and the chlorine concentration change rate averaged 2.5 × 10$^{-4}$ mg Cl$_2$/L·min (5.3 × 10$^{-5}$ - 2 × 10$^{-4}$ mg Cl$_2$/L·min) (Fig. 6.2(a)). Monochloramine penetrated into the biofilm surface layer 49 times faster than chlorine within the nitrifying biofilm.

Figure 6.6 shows a progression of CLSM images of the biofilm during chlorine disinfection (Phase 1: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.7 mg Cl$_2$/L monochloramine). The results were consistent with the observed disinfection performances (i.e. inactivation) for the respective contact times. However, monochloramine was not able to entirely damage the cells in the biofilm; even after 24 hrs of treatment, monochloramine penetrated fully with 96% of the bulk concentration throughout the biofilm.

Although monochloramine is a weaker disinfectant than chlorine when tested against bacteria cultured in suspension (Griebe et al, 1994), Comparison of DO microprofiles, which were measured during the chlorine disinfection (Fig. 6.2.(b)) and monochloramine disinfection (Fig. 6.5.(b)) showed that the cessation of aerobic microbial activity was more complete under chloramination. It appears that the biofilm may have a higher free chlorine demand than a monochloramine demand on the cessation of aerobic microbial activity. If microorganisms in a biofilm were just as susceptible as suspended planktonic
cells and the only protection in the biofilm environment was due to the retarded delivery of an antimicrobial agent, then killing of the biofilm should ensue once a fatal dose of antimicrobial had fully penetrated (Stewart et al. 2001). However, in this study, poor biofilm killing of the membrane compromised cells was observed using CLSM despite effective penetration of monochloramine at 2.74 mg Cl₂/L into the biofilm for 24 hours, as shown in Figure 6.6(c). Either more time (i.e. > 48 hrs) with the same concentration or a higher concentration (i.e. between 4.2 mg Cl₂/L and 10mg Cl₂/L) of monochloramine was required to entirely damage cells membranes in the biofilm, as shown in Figure 6.7 (a), (b), and (c).

6.4.3. Chlorination after monochloramine disinfection

After chloramination, the feed solution was changed from monochloramine to chlorine of the same concentration (2.8 mg/L as Cl₂). Fig. 6.8 presents evidence for diffusion limitation of chlorine action against a nitrifying biofilm matrix from the surface, even though monochloramine had already penetrated. Figure 6.9 shows the CLSM images of the biofilm at 24 hr chlorination (end of Phase 2 in Fig. 6.8) after 24hr monochloramine disinfection. It was observed that bacteria in the biofilm at the surface were entirely dead based on fluorescent red, indicating membrane compromised cells. It seems that monochloramine penetrates the biofilm matrix because of its lower reactivity and affects the whole biofilm system over time. On the other hand, when free chlorine, which is very strong oxidant, is used, it seems that once free chlorine oxidizes the organic matter in the biofilm, the reaction stops and it can diffuse further as shown in Figure 6.4.(a). It appears that chlorine may act different actions of chlorine at the diffusion layer
and surface of biofilms than with monochloramine. One possible explanation of the
different action of chlorine penetration is the biofilm sloughing or detachment from the
chlorination. The oxidization from free chlorine may change the biofilm morphology
(e.g. sloughing or detachment) and allow the chlorine to penetrate slowly into the biofilm
with time. Griebe et al. (1994) indicated the reduced areal density of viable cells from
detachment or sloughing when using chlorine. van der Wende (1989) also reported
significant sloughing or detachment during the disinfection with free chlorine, whereas
when applying monochloramine, no cell detachment was monitored. Some biofilm
sloughing was observed during the chlorine experiment (Phase 2) by naked eye.
However, more detail experiment which is focused on the biofilm sloughing from the
chlorination will be needed in the future.

6.4.4. Disinfectant penetration comparison with high concentration

Disinfections using a higher dose (800 mg Cl₂/L) with monochloramine and chlorine
were also studied and compared by measuring disinfectant microprofiles with time.
Figure 6.10 shows monochloramine penetration into the biofilm over time during
monochloramine disinfection (deoxygenated 5 mM buffer solution, pH 8.0, 23°C, and
800 mg Cl₂/L monochloramine). Figure 6.11 shows chlorine penetration into the biofilm
over time during chlorine disinfection (deoxygenated 5 mM buffer solution, pH 7.0, 23°C,
and 800 mg Cl₂/L chlorine). The microprofiles show the presence of a significant
diffusion boundary layer, with a thickness of 600 to 1,000 µm. Monochloramine
penetrated into a 830 µm thick biofilm slice to about 74% of its initial concentration
within 90 min after disinfection and to 79% within 2 hours. Chlorine penetrated only
0.4% into the 1,300 µm thick biofilm to around a 700 - 1,000 µm penetration depth, and this did not increase as much in the biofilm at the same time interval as did monochloramine. Chlorine was consumed continuously from the diffusion layer of the biofilm (~1,000 µm) during the experiment. Chlorine penetration microprofiles showed the slowly preceded penetration of chlorine, indicating that the free chlorine reacts with organic matter in the biofilm matrix because it is very strong oxidant and, once the oxidization of chlorine is completed, it can diffuse further. Conversely, monochloramine showed a well-defined continuous progression of penetration over time, indicating that the diffusion of monochloramine was much faster than the reaction rate with biofilm. The monochloramine concentration change rate at the surface averaged 5.45 mg Cl₂/L·min (0.56 – 14.72 mg Cl₂/L·min), calculated from Figure 6.9. The chlorine concentration change rate at the surface averaged 0.14 mg Cl₂/L·min (0.13 – 0.15 mg Cl₂/L·min), determined from Figure 6.10(b). Monochloramine penetrated into the biofilm surface layer 39 times faster than did chlorine in this experiment, and was shown to have better penetration into the biofilm compared to chlorine.

6.4.5. Reaction-diffusion interaction comparison between chlorine and monochloramine

A highly reactive antimicrobial agent (i.e. chlorine) is known to be chemically consumed on its way into the biofilm, depleting the neutralizing capacity at the surface of the biofilm before it can penetrate into deeper layers of the biofilm (Stewart et al. 2001). Our results are consistent with those of previous investigations about the more retarded penetration of hypochlorite than that of an antimicrobial agent which has a slower
reaction rate (de Beer et al. 1994; Chen et al. 1996; Xu et al 1996; Stewart et al. 2001). Monochloramine penetrated biofilms more rapidly than chlorine due to a slower reaction rate with biofilm constituents.

Figure 6.12 shows a comparison of penetration into the biofilms between chlorine and monochloramine. The ratio of the disinfectant concentration near the substratum (C_{biofilm}) to the disinfectant concentration in the bulk (C_{bulk}) was plotted to facilitate comparison of the extent of penetration. If this ratio was 0, there was no penetration and if the ratio was 1, penetration was complete. Monochloramine penetrated into biofilm more rapidly than chlorine. The time required to attain 50% of the monochloramine biofilm penetration near the substratum was average less than 2 hours in this research. On the other hand, chlorine showed only a maximum of 9% penetration during the times when experiments were conducted, except the experiment with 9.6 mg Cl\textsubscript{2}/L of chlorine after 24 hrs chlorine (2.6 mg Cl\textsubscript{2}/L) disinfection; here, 35.4% chlorine penetrated into the bottom of the biofilm. Overall, chlorine showed the penetration limitation into the biofilm and monochloramine showed greater penetration than chlorine.

6.5. Conclusions

The disinfectant penetration profiles for monochloramine and chlorine into biofilms were directly measured and compared with DO microelectrodes and CLSM observations were made, correlating penetration with aerobic microbial activity and viability. Monochloramine showed greater penetration compared to chlorine, indicating the apparent cessation of aerobic activity within the nitrifying biofilm upon the administration of chloramine disinfectant. Monochloramine penetrated into the biofilm
surface layer 49 times faster than chlorine within the nitrifying biofilm and 39 times faster in the multi-species biofilm than did chlorine. On the other hand, free chlorine seems to be consumed by oxidizing the organic matter in path of the biofilm, then diffuse further, indicating very slow chlorine penetration. However, CLSM image of membrane compromised cells in biofilm after chlorination confirmed that free chlorine is much stronger than monochloramine in disinfection. Comparison of DO microprofiles, which were measured during the chlorine disinfection and monochloramine disinfection respectively, showed that the cessation of aerobic microbial activity was more complete under chloramination. The time required to attain 50% of the monochloramine biofilm penetration near the substratum was average less than 2 hours in this research. On the other hand, chlorine showed only a maximum of 9% penetration during the times when experiments were conducted, except the experiment with 9.6 mg Cl₂/L of chlorine after 24 hrs chlorine (2.6 mg Cl₂/L) disinfection; here, 35.4% chlorine penetrated into the bottom of the biofilm. The results in this study support the hypothesis (3) and (4) that monochloramine penetrates more quickly and further into the biofilm than chlorine and monochloramine and free chlorine act differently at surfaces of biofilm composed of various bacteria, and provides useful information for use of disinfectants for biofilm control.
Figure 6.1 Experimental setup for microprofiling with multi-species biofilm
Figure 6.2  Microprofiles of chlorine and DO during chlorine disinfection (Phase 1: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.6 mg Cl₂/L chlorine)
(a) Intact biofilm (before disinfection) (Z stack: 183.17 μm)

(b) 7 hour after chlorine disinfection (Z stack: 128.89 μm)
Figure 6.3  Progression of CLSM images of the biofilm during chlorine disinfection (Phase 1: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.6 mg Cl₂/L chlorine). Green and red coloring indicates live and dead cells, respectively.
Figure 6.4  Microprofiles of chlorine and DO during chlorine disinfection (Phase 2: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 9.6 mg Cl$_2$/L chlorine)
Figure 6.5 Microprofiles of monochloramine and DO during monochloramine disinfection (Phase 1: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.7 mg Cl₂/L monochloramine)
(a) Intact biofilm (before disinfection) (Z stack: 33.92 μm)

(b) 2 hour after monochloramine disinfection (Z stack: 61.06 μm)
Figure 6.6 Progression of CLSM images of the biofilm during monochloramine disinfection (Phase 1: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.7 mg Cl₂/L monochloramine). Green and red coloring indicates live and dead cells, respectively.
(a) 48 hr monochloramine treatment (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.7 mg Cl₂/L monochloramine) (Z stack: 101.7 μm).

(b) 24 hr monochloramine treatment (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 10 mg Cl₂/L monochloramine) (Z stack: 81.4 μm)
(c) 24 hr monochloramine treatment (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 20 mg Cl₂/L monochloramine) (Z stack: 183.3 μm)

Figure 6.7 CLSM images of the biofilm after treatment with different contact time and disinfectant concentrations for entirely damaging cells membranes. Green and red coloring indicates live and dead cells, respectively.
Figure 6.8 Microprofiles of chlorine and DO during chlorine disinfection after monochloramine fully penetration (Phase 2: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.8 mg Cl₂/L chlorine)
Figure 6.9 CLSM images of the biofilm (Z stack: 122.1 μm): 24hr chlorination after 24hr monochloramine disinfection (Phase 2: 5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 2.8 mg Cl₂/L chlorine). Green and red coloring indicates live and dead cells, respectively.
Figure 6.10  Monochloramine concentration microprofile (deoxygenated 5 mM buffer solution, pH 8.0, 23°C, and 800 mg Cl₂/L monochloramine)
Figure 6.11  Chlorine concentration microprofile (deoxygenated 5 mM buffer solution, pH 7.0, 23°C, and 800 mg Cl₂/L chlorine)
Figure 6.12 Comparison of disinfectant penetration between monochloramine and chlorine

1) $C_{\text{biofilm}}$: disinfectant concentration inside biofilm at 200 $\mu$m inside the nitrifying biofilm with 250 - 350 $\mu$m thickness or 700 $\mu$m inside the multi-species biofilm with 830 - 1,300 $\mu$m thickness.

2) $C_{\text{bulk}}$: disinfectant concentration in the bulk at 1,000 $\mu$m from the surface of the nitrifying biofilm with 250 - 350 $\mu$m thickness or at 3,000 $\mu$m from the surface of the multi-species biofilm with 830 - 1,300 $\mu$m thickness.
Chapter 7. The Effects of Phosphate on Nitrifying Biofilms and Monochloramine Penetration in the Drinking Water Distribution System

7.1. Abstract

Nitrification is a potential problem for utilities that have switched their secondary disinfectant from chlorine to monochloramine due to the trihalomethanes (THM) and haloacetic acids (HAA) regulations. Meanwhile, orthophosphate (PO$_4$$_{3-}$) plays an important role in corrosion control, combining with lead and copper in plumbing materials; it is recommended to maintain a phosphate residual of at least 0.5 mg P/L and, if possible, a residual of 1 mg P/L is preferable. However, relatively little is known about the effect of phosphate on nitrifying biofilm in chloraminated drinking water distribution systems when it comes to addition of phosphate to the water distribution system. This study aimed at evaluating the effect of phosphate as an anti-corrosion chemical (corrosion inhibitor) on nitrifying bacteria biofilms in a simulated model drinking water distribution system with macro- and micro-monitoring and confocal laser scanning microscopy (CLSM) observations. Three annular biofilm reactors were operated with different phosphate concentrations (0, 1, and 12 mg P/L) over 5 months. Monochloramine penetrations with and without the presence of phosphate were also compared using microelectrodes and LIVE/DEAD® BacLight™. Phosphate was found to act positively on biofilm development and microbial activity (i.e. nitrification) in the long term. Phosphate microprofiles showed that phosphate contents in the biofilm was independent on the nitrifying activity. Low availability of phosphorus seemed to change biofilm
structure at the biofilm surface. Phosphate did not affect the monochloramine penetration and monochloramine fully penetrated into the nitrifying biofilm within 24 hours both with and without phosphate. However, DO penetration was retarded in the presence of phosphate for a while. CLSM observation showed bacteria with intact cell membranes were observed even after monochloramine fully penetration, indicating monochloramine penetration did not equal a loss in viability. The phosphate concentration was decreased after the monochloramine disinfection in the presence of phosphate due to the phosphate diffusion out of the biofilm. These results provide a better understanding of the relationship between phosphate and nitrifying biofilm in chloraminated drinking water distribution systems, allowing better prevention and control strategy of nitrification in the presence of phosphate in practice.

**Keywords:** Biofilm; corrosion inhibitor; confocal laser scanning microscopy (CLSM); LIVE/DEAD BacLight; microelectrode; monochloramine; nitrification; nitrifying biofilm; orthophosphate

### 7.2. Introduction

The implementation of increasingly stringent regulations for trihalomethanes (THM) and haloacetic acids (HAA) in the United States has resulted in an increasing use of chloramine within the past two decades as a secondary disinfectant in the drinking water treatment industry (Nikolaou et al. 2007). A recent survey in the US showed that 30% of water utilities serving populations of more than 10,000 people use chloramines for secondary disinfection in order to meet regulations imposed by the United States Environmental Protection Agency (USEPA) under the Stage 1 and Stage 2 Disinfectants/
Disinfection Byproducts Rule (D/DBPR) (AWWA 2000). Along with the addition of chloramines comes the risk of nitrification in the distribution system (AWWA 2006; Kirmeyer et al. 2004; Kirmeyer et al. 1995). The ammonia released from chloramine decay (i.e. autodecomposition, oxidation of natural organic matter (NOM), corrosion, catalysis at pipe surface, and oxidation of nitrite) can support autotrophic microbial activity (Zhang et al. 2008). Based on utility surveys, 30 - 63 % of utilities using monochloramine as a disinfectant have experienced nitrification episodes (Wilczak et al. 1996; Odell et al. 1996; Seidel et al. 2005). Nitrification in drinking water distribution systems may result in degradation of water quality and subsequent non-compliance with existing regulations (AWWA 2006).

Organic carbon and organic acids produced via autotrophic microbial nitrification can stimulate growth of heterotrophic bacteria, contribute to loss of disinfectant, and create problems with lead and copper contamination from corrosion (Zhang et al. 2008). In accordance with Lead and Copper Control Strategies (USEPA 2003), it is indicated that orthophosphate ($\text{PO}_4^{3-}$) as a corrosion control treatment chemical should be maintained at a residual of at least 0.5 mg/L as phosphate (P) and, if possible, a residual of 1 mg/L as P is preferable. Although Zhang et al. (2008) proposed that nitrification in polyvinyl chloride (PVC) premise plumbing is insensitive to phosphate concentrations of 5 - 1,000 ppb, some researchers indicate that phosphorus can affect microbial growth in drinking water (Miettinen et al. 1997; Keinänen et al. 2002, Lehtola et al. 2002), and nitrifying activity was immediately enhanced after addition of phosphate for the purpose of ammonia removal in one water treatment plant (Takayuki et al. 2002). Currently, nitrification control is a major issue in practice and is likely to become increasingly
important as chloramine use increases in drinking water distribution systems. In addition to potentially increasing nitrification, phosphate (i.e. orthophosphate (PO$_4^{3-}$)) as a corrosion inhibitor can aggravate water quality. Relatively little is known about the effect of phosphate on nitrifying biofilm in chloraminated drinking water distribution systems with respect to the addition of phosphate to the water distribution system.

This study was therefore carried using annular biofilm reactors, under controlled laboratory conditions, to determine the effect of phosphate on nitrifying biofilm development and nitrification in a simulated water distribution system with various phosphate concentrations. The effect of various phosphate concentrations on nitrifying biofilm structure changes were also evaluated and the monochloramine penetration was compared with and without the presence of an orthophosphate residual using microelectrodes and confocal laser scanning microscopy (CLSM) with LIVE/DEAD® BacLight™.

### 7.3. Experimental materials and methods

#### 7.3.1. Annular biofilm reactor

Three annular biofilm reactors (BioSurface Technologies Corp., Bozeman, MT, Model 1320 LJ) were used to develop nitrifying biofilms in various phosphate concentrations (i.e. 0 mg P/L, 1.0 mg P/L, and 12 mg P/L), as shown in Figure 7.1, with temperature, substrate and shear stress conditions of a typical drinking water distribution system. The reactors consisted of an outer glass cylinder, and a rotating inner polycarbonate cylinder with space for twenty flush-mounted slides. Twenty polycarbonate slides in each reactor, providing an attachment surface for biofilm samples,
were used to investigate the effect of phosphate on the biofilms without other reactions with pipe material. Shear stress was applied to the slide surface by setting the inner cylinder rotation to 100 rpm for all experiments, which produces shear on the inner drum similar to a 30.5 cm/s (1ft/s) flow in a 10.2-cm (4-in.) pipe (Szabo et al. 2006). This shear is valid for a smooth surface on the inner drum of the reactor, and the reported shear should be considered approximate. The total volume of the reactors was maintained at 750 mL by setting the height of the effluent tubing and adjusting the water level. Biofilms were grown both on the outer surface of the inner drum, mainly on the surfaces of the polycarbonate slides, and on the inner surface of the stationary outer drum. The reactors were covered with aluminum foil to prevent light penetration and growth of algae. Tap water from the City of Cincinnati was fed through a granular activated carbon (GAC) column to remove residual chlorine before being fed to the mixed cultures. This chlorinated water was transferred to the reactor by a peristaltic pump (Cole-Parmer, Vernon, IL). The hydraulic residence time (HRT) of the reactor was set at 6 hrs to simulate nitrification occurrence, considering the residence time or water age in the distribution system. Air was injected at a relatively low flow rate into the headspace of the reactors by adjusting the water level in the reactors to maintain the dissolved oxygen (DO) concentration (1 - 3 mg/L) in the bulk solution. Nutrients and alkalinity were injected through a tubing (HV-06424-13, Cole Parmer, Vernon Hills, IL) with a small inside diameter (0.8mm). In the initial periods of the operation, the tubing was connected directly with the alkalinity inlet line and the nutrient inlet line. This line occasionally got clogged due to the lime which was generated from the high concentration of sodium bicarbonate. This problem was solved by setting up the inlet line separately and
increasing the flow rate (0.223 mL/min) with a lower concentrated sodium bicarbonate solution.

7.3.2. Inoculation and nitrifying biofilm development

Mixed cultures were acquired from utilities experiencing nitrification episodes in chloraminated drinking water distribution systems. This culture was seeded into each of the annular reactors containing the appropriate media and operated in batch mode (i.e. no influent or effluent flow) at pH 8.0 and 23°C until nitrification was detected. The media initially contained 250 mg N/L of ammonia. These reactors were monitored for ammonia removal while maintaining DO (by air addition) and pH (by 5% w/v NaHCO₃ addition) at appropriate levels. No other additions (i.e. carbon, trace mineral) were made to the reactors during this phase of operation. Once nitrification was monitored by a drop in the ammonia level (i.e. below 50 mg N/L after 1 week of the batch operation), the reactor was operated in a continuous mode with a relatively low influent ammonia concentration, which then increased over time. (i.e. from 20 mg N/L to 200 mg N/L). In the initial condition, synthesized water contained 7.14 mM (NH₄)₂SO₄, 0.4 mM KH₂PO₄, 1.0 mM KCl, 0.2 mM MgSO₄·7H₂O, 1.0 mM CaCl₂·2H₂O, 10 mM NaCl, 2.0 mL/L organic cocktail stock solution (4,000 mg C/L), and 1.0 mL/L trace elements stock solution. The organic carbon cocktail source consisted of 0.088 M ethyl alcohol (C₂H₆O), 0.098 M propionaldehyde (C₃H₆O), 0.525 M oxalic acid (C₂H₂O₄), 0.158 M pyruvic acid (C₃H₄O₃), and 0.131M acetic acid (C₂H₄O₂). The five organic compounds represent major classes of organic compounds which are found in drinking water that are relatively nonreactive with disinfectants (Gagnon et al. 2004). Each organic compound was added
to the cocktail in equimolar concentrations based on oxygen demand. This carbon super-
stock solution was adjusted to ~pH 7 with concentrated (i.e. > 6N) NaOH. The trace
elements solution contained 0.020 mM MnCl₂·4H₂O, 0.8 mM H₃BO₃, 0.15 mM
ZnSO₄·7H₂O, 0.03 mM (NH₄)₆MnO₂₄·4H₂O, 3.5 mM FeSO₄·7H₂O, 0.1mM
CuCl₂·2H₂O, and 0.025 N HCl (6 normal). Concentrated NaHCO₃ was continuously fed
to the reactors separately to adjust the pH to pH 7.5 – 8.0 in the bulk liquid in the reactor.
Carbon concentration added to the reactors was 8.0 mg C/L and, therefore, the N:C ratio,
N:P ratio, and C:P ratio were 25, 16, and 0.65, respectively. After steady-state
nitrification was established, the phosphate concentration was adjusted to three different
phosphate concentrations (i.e. reactor 1: 0 mg P/L, reactor 2: 1.0 mg P/L, reactor 3: 12
mg P/L) by removing phosphate in the original feed solution and separately injecting
each appropriate amount of phosphate to the bulk phase of reactor 2 and reactor 3 with
phosphate stock solution (2,500 mg P/L) with a syringe pump (Figure 7.1) at rate of 0.45
ml/hr. Micro-cassette pumps (IP 16, Ismatec SA, Labortechnik, Switzerland) with 16
channels were used to control the flow of nutrients and alkalinity. Ammonia, nitrite,
nitrate, phosphorus, DO and pH monitoring in the reactor was conducted continuously
during the whole experiments. The bulk solution in each reactor was analyzed for
ammonia, nitrate, nitrite, and phosphorus using Hach methods (i.e. ammonia: Hach-8038
(Nessler Method), nitrite: Hach-8153 (Ferrous Sulfate Method), nitrate: Hach-8039
(Cadmium Reduction Method), phosphorus: Hach-8048 (Ascorbic Acid Method)) using a
DR/2010 spectrophotometer (Hach Co.). DO was measured using an OM-4 oxygen
meter (Microelectrodes, Inc., Bedford, NH). pH was measured using a pH/ATC
combination electrode (Cat. No. 13-620-112, Accumet) with a pH meter (Model 15, Accumet).

### 7.3.3. Microelectrode preparation and measurements

#### 7.3.3.1. Phosphate microelectrode

Details of the sensing mechanism, fabrication procedures and calibration of phosphate microelectrodes were described previously in Chapter 3.

#### 7.3.3.2. Chlorine microelectrodes

A chlorine microelectrode was used to measure the monochloramine penetration microprofiles during monochloramine disinfection. Details of the sensing mechanism, fabrication procedures and calibration of chlorine microelectrodes for monochloramine measurement were described previously in Chapter 4.

#### 7.3.3.3. Ion-selective microelectrodes (pH, NO₃⁻, NH₄⁺)

These microelectrodes are neutral carrier-based liquid membrane ion-selective microelectrodes. Their microelectrode structures, fabrication procedures and calibration methods are similar to each other, but with different filling and standard solutions. The ion exchange microelectrode for ammonium was fabricated with a neutral carrier (electrically neutral ionophore) and a borosilicate glass micropipette (Sutter Instrument Company, BF 120-69-15, ID: 0.69 cm, OD: 1.2 mm, length: 15 cm) (Li, 2001). After filling the micropipette with the internal reference solution (electrolyte solution) composed of 0.01 M NH₄Cl, the liquid membrane (ammonium ionophore I-cocktail A
Selectophore®, No.09879, Fluka) was filled into the salinized tip of the microelectrode by capillary force. The fabricated ammonium microelectrode was calibrated in various concentrations of ammonium from $10^{-5}$ M to $10^{-2}$ M. The pH microelectrode and nitrate microelectrode were fabricated with the same procedures as for the ammonium microelectrode except that a different ion-selective cocktail was used for each liquid membrane (i.e. pH microelectrode: hydrogen ionophore I-cocktail B Selectophore®, No. 95293, Fluka, nitrate microelectrode: nitrate ionophore-cocktail A Selectophore®, No. 72549, Fluka). The details for fabrication were patterned after Yu (2000), Li (2001), and Peng (2007).

### 7.3.3.4. DO microelectrodes

The DO microelectrode is a polarographic recessed cathode gold electrode. The polarization voltage is set at -750 mV by using the power source. The method of fabrication followed Peng (2007), Fu (1993), Zhang (1994), and Yu (2000). The tip diameters ranged from 3 to 15 μm. The oxygen microelectrode was used as the working (cathode) electrode, while a separate milli-reference electrode was placed in proximity during calibration and measurement.

### 7.3.3.5. Microprofile measurements

After nitrification was achieved in the reactors, in situ nitrifying activities were measured with ammonium pH, nitrate and phosphate microelectrodes. A polycarbonate slide holding well-grown nitrifying biofilm was taken from each annular biofilm reactor (reactors 1, 2, and 3) two months after changing the phosphate concentrations. This slide
was inserted into the flow chamber, which has a small reservoir with two inlet holes to prevent flow disturbance or short-cutting before the main measurement part of the flow cell (Figure 7.2(a)). This flow chamber was fed with the appropriate medium continuously with an average velocity of 15 mL/min at 23°C, and air was injected into the medium to maintain 8.3 mg/L DO. The following medium composition was used as the feed solution for microprofiling measurements: 0.15 mM (NH₄)₂SO₄, 8×10⁻³ mM NaNO₃, 8×10⁻³ mM NaH₂PO₄·H₂O, 4×10⁻³ mM MgSO₄·7H₂O, 0.02 mM CaCl₂·2H₂O, 0.2 mM NaCl, 2.5g/L NaHCO₃, and 0.02 mL/L trace elements stock solution. This medium was adjusted to pH 8.0 by adding 0.25N NaOH. Potassium ions were not added to avoid interference with the ammonium microelectrode (de Beer et al. 1993). The biofilm samples taken from the reactor were acclimated in the medium for an hour before the microprofile measurements to ensure that steady state profiles were obtained (Okabe et al. 1999). Under the stereomicroscope (Nikon SMZ2T), biofilm was examined and an appropriate position for microprofiling was selected. Then, an old microelectrode was used to measure the biofilm thickness. The biofilm thicknesses used in this experiment were between 350 – 450 µm.

A newly fabricated microelectrode, which had already been calibrated in the medium used for the measurements, was fixed to the microelectrode holder and this holder was moved by a three-dimensional micromanipulator (World Precision Instruments, M3301). By controlling this micromanipulator and monitoring the monitor which was connected to a charge coupled device (CCD) camera (Javelin JE3662HR) and the stereomicroscope, the tip of the microelectrode was initially positioned at 1,000 µm above the biofilm-water interface. Figure 7.2(b) shows the microprofiling experimental setup. For well defined
microprofiles, the microelectrode positioning to the well-shaped biofilm structure was very important as was the use of well-performing microelectrodes. Figure 7.2(c) shows the CLSM image of representative microelectrode positioning. The measurement was performed at intervals of 50 to 100 μm from this point (i.e. 1,000 μm above the biofilm surface). Microprofiles were measured three times at different positions in the biofilms for each species and set of conditions (i.e. reactors 1, 2, and 3). All measurements were conducted inside a Faraday cage (Technical Manufacturing Corporation [TMC], Peabody, Massachusetts) to minimize signal noise. DO and monochloramine concentrations were measured using a Chemical Microsensor II (Diamond General Corp., Product No 1231) potentiostat by obtaining output picoampere current signals (pA). pH, ammonium, nitrate, and phosphate concentrations were measured using a pH meter (model 215, Denver Instruments, Denver, Colorado) by obtaining potentiometric signals (mV). Each microelectrode was calibrated before and after each constituent concentration microprofile measurement to ensure the stability and good performance of the electrode during experiments.

7.3.4. Monochloramine disinfection experiments

Two monochloramine disinfection experiments were conducted to investigate the effect of phosphate on monochloramine penetration within nitrifying biofilms. One experiment was conducted with only monochloramine and the other experiment was conducted in the presence of both monochloramine and phosphate (1 mg P/L). 4.6 - 5.5 mg Cl₂/L monochloramine (4:1 Cl₂:N) was prepared in 5 mM pH 8.0 buffer solution (1510-4L, Aqua Solutions, Inc., Deer Park, TX) by adding an calculated amount of a
sodium hypochlorite solution (5% available chlorine, No.:7495.5-32, Ricca Chemical, Fisher Scientific, Pittsburgh, PA) and ammonia stock solution (100 mg N/L). This monochloramine solution was stored at 23°C for several hours before the experiments. In the beginning of each experiment, air was injected to maintain 8.3 mg/L of DO concentration in the monochloramine feed solution. Preliminary experiments showed that the air bubbling into the solution did not affect the monochloramine concentration within the disinfection time tested (i.e. 24 hours). For each experiment, a polycarbonate slide containing a well-developed nitrifying biofilm was taken from reactor 3. This slide was cut into four pieces from its original 15 cm long slide. These pieces were used for microprofiling and CLSM imaging with fluorescent stain. LIVE/DEAD® BacLight™ (L7012, Molecular Probes, Inc, Eugene, OR) was used to stain live (i.e. bacteria with intact cell membranes) and dead cells (i.e. bacteria with damaged cell membranes) in the biofilm before and after monochloramine disinfection, and disinfection results were determined with Confocal Laser Scanning Microscopy (Zeiss LSM 510 CLSM). Three CLSM images were taken: 1) intact sample, which represents live cells; 2) sample taken at a time when monochloramine penetrated with half of the initial concentration into the bottom of the biofilm; and 3) sample with full monochloramine penetration. The remaining biofilm piece was used for monochloramine, DO, and phosphate microprofiling with time. DO and monochloramine concentration microprofiles were measured at the same time intervals. Phosphate concentration microprofiles were measured before and after monochloramine disinfection.

Monochloramine concentration in the bulk solution was measured using colorimetric test kits (Total chlorine: Hach-8167; Monochloramine: Hach-10200) and a DR/2010
spectrophotometer (Hach Co.). Blank microprofiles were measured with new (i.e. intact) polycarbonate slides which had no biofilm on them. There was no signal change in the bulk regardless of the distance from the slide (data not shown), indicating that a polycarbonate slide did not respond to monochloramine or affect monochloramine concentration microprofiles.

7.3.5. CLSM observation

Visualization of biofilm inactivation by the method of Tachikawa et al. (2005) provided another option to assess disinfection by in situ staining with subsequent viewing with confocal scanning laser microscopy (CSLM) in order to visualize the spatial effect of the disinfectant on bacterial viability. In this chapter, LIVE/DEAD® BacLight™, which is a culture-independent membrane integrity based technique, was used to evaluate live and dead cells within nitrifying biofilm before and after monochloramine disinfection. Bacteria with intact cell membrane (fluorescent green) are described to be alive, whereas bacteria with damaged membranes (fluorescent red) are described to be dead. Therefore, a cell was considered viable or nonviable based on the ability of propidium iodide (PI) to penetrate its membrane and subsequent processing according to the method of LIVE/DEAD® BacLight™. The Zeiss LSM 510 laser scanning confocal microscope was used to observe and examine biofilm viability and structure. A piece of biofilm on a polycarbonate slide was stained with the LIVE/DEAD® BacLight™ viability kit. Staining was conducted by adding the live and dead stains, which were mixed in distilled water, to the biofilm sample. 3 μl/ml of SYTO® 9 and 9 μl/ml of propidium iodide were added to 1,000 μl of DI water. 300 μl of mixed stain solution was added to
each slide. Then the stained biofilm sample was incubated in darkness (with aluminum foil) for 15 minutes at room temperature before imaging with the confocal microscope. The treated sample was immersed into DI water (50 mL) to remove excess dye and was used for imaging the biofilm surface. The excitation/emission maxima for dyes are about 488/505-550 nm for SYTO® 9, the green-fluorescent nucleic acid stain for live cells, and 543/585 nm for propidium iodide, the red-fluorescent nucleic acid stain for dead cells. The objective of the Plan-Neofluar 10×/0.3 microscope was used for observation. Two filters (i.e. Ch1 (red): LP 585; Ch2 (green): BP 505-550) were used. Computer image micrographs were obtained, with the biofilm depth adjusted by using a Zeiss LSM Image Browser, Version 4.4.4.241. For a dead cell control, one piece of biofilm slide was treated by boiling at 80°C for 2 hours and used in every CLSM observation. All CLSM images were only obtained to about half way into the biofilm because of the limitation of dye penetration and CLSM observation depth.

7.4. Results and discussion

7.4.1. Nitrification occurrence monitoring

After the inoculation of nitrifying bacteria from the distribution systems experiencing nitrification, the three reactors were operated under the same conditions for 4 months (Phase 1 of each reactor in Table 7.1), then operated for 4 months with three difference influent phosphorus concentrations (i.e. 0, 1.0, and 12 mg P/L) (Phase 2). The annular biofilm reactor performance was evaluated by comparing the key components in the bulk solution of each reactor. Table 7.1 shows the reactor performance results during the experiments. The pH of the influent was maintained in the range of 7.5 - 8.0 at 23 °C
room temperature during the whole experiment, based on the lead and copper control strategies (USEPA, 2003) in which the pH should be maintained within the range of 7.2 - 7.8 when using orthophosphate as a corrosion inhibitor. In Phase 1, nitrification was successfully achieved, and biofilms in all three reactors were well developed with thicknesses of 150 - 350 μm and with relatively even distributions across the polycarbonate slides after 2 months. Since the biofilm had grown under shear stress and high flow velocity, it was firm and had a low susceptibility for sloughing. In Phase 2, well-defined nitrification was observed in all three reactors, even though phosphorus concentrations in the influent were different. Microbially available organic phosphorus can be calculated to be 30% in reactor 2 and 46% in reactor 3. In reactor 1, phosphorus (0 - 2.8 mg P/L) in the bulk appears to be formed due to the phosphate diffusion from the biofilm in the phosphate limited condition. These different phosphate utilization results regardless with nitrification in three reactors show the possibility of changes of heterotrophic microbial communities, even though the autotrophic microbial communities were predominantly active in these systems.

From the operational results presented in Table 7.1, nitrification, when well established, was not affected by the phosphorus concentration, and there were no significant changes in nitrification among the three reactors during the operation. However, based on the ammonia-N, nitrite-N, and nitrate-N monitoring, it appears that, as phosphate concentration increased, more ammonia was consumed or was converted to nitrite and nitrate. This result can be explained by the different biofilm thicknesses in the three reactors. In this phase, the biofilm thickness was observed and measured with a used microelectrode. The biofilm thickness decreased with time when the phosphate was
limited. Reactor 1 had less thick biofilms (150 – 350 μm) on the slides than did reactor 2 (200 – 400 μm) or reactor 3 (250 – 400 μm) during the operation periods (4 months). Therefore, in the long term, phosphate is expected to act positively on biofilm development and microbial activity (i.e. nitrification).

During the reactor operation, the loss of nitrogen may have been caused by denitrification and/or incorporation into biomass during cell synthesis. It was reported that autotrophic ammonia-oxidizers can perform aerobic/anoxic denitrification with nitrite as the electron acceptor and ammonia as the electron donor (Helmer et al. 1999).

7.4.2. Effects of phosphate on nitrification determined from micro-profiling

The microprofiles were measured during steady-state of Phase 2; that is, two months after changing the phosphate concentration. Figure 7.3 shows the microprofiles of dissolved oxygen (DO), ammonium, pH, nitrate, and phosphate in the nitrifying biofilm in the three reactors. Constituent concentration microprofiles did not show any significant differences among the three reactors. The three reactors showed similar aerobic microbial activities, except for phosphate due to the different phosphate influent concentrations. All DO microprofiles indicated that the oxygen diffusion boundary layer thickness was around 500 μm. Reactor 2 biofilm had only an aerobic zone, as oxygen totally diffused throughout the biofilm (Fig. 7.3(b)). DO profiles in Figure 7.3(c) can be divided into two parts, indicating an aerobic zone and an anoxic zone, because of the limitation of oxygen diffusion through the extracellular polymeric substances and utilization of the oxygen by the microbial activity. These concentration profiles (Fig. 7.3)
describe well-defined nitrifying biofilm microbial activities, and CLSM images (Fig. 7.4, 7.5, and 7.6) confirmed that the cells were essentially all alive in the biofilm. Ammonium decreased about 30 – 36% in the biofilm. pH decreased because of the alkalinity consumption by nitrification and nitrate was produced inside the biofilm. These microprofiles all indicated that intensive ammonia-oxidizing activity was occurred at the biofilm surface, with an average 250 μm thick ammonia diffusion boundary layer; the nitrite-oxidizing activity increased with depth into the biofilm. This finding is in agreement with the report by Okabe et al. (1999). The results of both microelectrode measurements and fluorescent in situ hybridization (FISH) showed the active ammonia-oxidizing zone was located in the outer part of the biofilm, while the active nitrite-oxidizing zone was located below the ammonia-oxidizing zone and overlapped the location of the nitrite-oxidizing bacteria (Okabe et al. 1999). This result could be possible because the $K_m$ for oxygen by ammonia oxidizers (16 μmol/L) is lower than the value by nitrite oxidizers (62 μmol/L) (Schramm et al. 1996). Therefore, it was concluded that the phosphate had no significant impact on the nitrifying microbial activity in the biofilm in the water distribution system biofilms in the range of 0 – 12 mg P/L in this measured period (i.e. 2 months after phosphate change).

Phosphate concentration was 0.7 mg/L (reactor 1), 2.7 mg/L (reactor 2), and 3.6 mg/L (reactor 3) at the surface of the biofilms, and 3.5 mg/L (reactor 1), 3.9 mg/L (reactor 2), and 10.7 mg/L (reactor 3) at the 300 μm depth into the biofilm. This result indicated that the phosphate contents in the biofilm were dependent on the microbial availability of phosphorus in the bulk when biofilm is formed, regardless of the nitrifying activity. The autotrophic microbial activity was not significantly different, while the phosphate
concentration inside the biofilm was different in each reactor, as shown in Fig. 7.3. The biofilm with a high phosphorus bulk condition (i.e. 12 mg P/L) had similar phosphate concentrations (i.e. 10.7 mg P/L) within the biofilm, whereas the biofilm in the phosphorus limited condition showed low phosphate concentrations within the biofilm. This change in phosphate concentration within the biofilm can be related to the change in extracellular polymeric substances (EPS) content in the biofilm because the phosphate is conserved in the EPS due to its good adsorptive properties (Loaëc et al. 1997). It appears that low phosphate concentrations at the surface of the biofilm in reactor 1 (Fig. 7.3(a)) may indicate low EPS contents in biofilm.

### 7.4.3. Effects of phosphate on nitrifying biofilm structure

Figures 7.4, 7.5, and 7.6 show the CLSM images of the nitrifying biofilm in reactors 1, 2, and 3, respectively. Again, the dye for Live/Dead cell could not be observed through the whole biofilm because of light penetration limitations. Nitrifying bacteria are well known to grow in clumps or aggregates attached to the pipe surface and this important feature indicates that nitrification in drinking water is essentially a biofilm phenomenon (AWWA 2006). This adhering feature also can be found in the marine environment where nitrifying bacteria are found in deposited sediments not in the water (AWWA 2006). This study also found that the biofilms have been formed originally with the aggregates or clumps of nitrifying bacteria as shown in Figure 7.6. These aggregates existed in the polycarbonate slides surface and outer case of the annular reactor by adhering and forming nitrifying biofilm. The CLSM image of the biofilm surface in reactor 1 (Fig. 7.4(a)) shows the thin and elongated aggregates of biofilm.
grown in the same direction as the flow (i.e. vertical direction), indicating different shapes with the images of reactors in the presence of phosphate (Fig. 7.5 and 7.6). Figure 7.4 (a) also shows that some parts of the biofilm surface seem to be detached.

This change of biofilm structure may be due to the low availability of phosphorus. Biofilm in reactor 1 visibly consisted of white clusters at the bottom of the biofilm with less dense than the biofilm of reactor 3. It seems that nitrifying bacteria colonies may need to change their biofilm structure morphology to contact more bulk phosphorus, under the condition of extremely low phosphate concentration, by adjusting their structure to have higher surface area.

There is another possible explanation of the structure change in the phosphate limited condition. It appears that this phosphate limit condition can result in the weakened cell structure and allow EPS to be detached from the biofilm. This detached EPS may explain that the low phosphate concentration within biofilm (Fig. 7.3(a)) and the bulk phosphate concentration of averaged 0.3 mg P/L (0 - 2.8 mg P/L) in the reactor 1 during the operation, even without phosphate in the feed (i.e. 0 mg P/L of influent phosphate concentration), as shown in Table 7.1. There are several reports of the relationship between EPS and phosphorus. Phosphorus was known to be conserved in the EPS because of its biosorption characteristics (Loaëc et al. 1997; Liu et al. 2006). Cloete et al. (2001) found that cell clusters with associated EPS contained between 57 – 59% phosphorus, and EPS alone contained between 27 – 30% phosphorus.

The structure change was observed mainly near the biofilm surface in the absence of the phosphate. The CLSM images in Figure 7.4(b), 7.5(b) and 7.6(b) indicate that the biofilm structure had similar shapes at a depth over half way into the Z stack. Phosphate
concentrations at the bottom of the biofilm are similar between reactor 1 (3.5 mg P/L) and 2 (3.9 mg P/L) because the structure did not change much, as shown in Fig. 7.4 (b) – 6(b).

7.4.4. Effects of phosphate on monochloramine penetration

The effects of simultaneous monochloramine and dissolved oxygen biofilm penetration are shown in Figure 7.7. Initially, the nitrifying biofilm was at steady-state in an oxygen saturated buffer (approx. 8.3 mg/L DO and pH 8.0), and then 4.6 mg/L (as Cl₂) monochloramine was added at time zero. Prior to chloramine addition, all available DO was utilized near the surface of the biofilm. Over the first few hours, as monochloramine penetrated into the biofilm, the aerobic activity of the biofilm decreased due to the disinfectant addition and the DO began to penetrate the biofilm as well. In about 27 hours, monochloramine and DO both fully penetrated the biofilm. Together these two figures showed the apparent cessation of aerobic activity within the nitrifying biofilm upon the administration of chloramine disinfectant. Figure 7.10 shows the progression of the biofilm during the monochloramine disinfection without phosphate. Before disinfection, intact biofilm was alive and in good shape (Fig. 7.10(a)). Figure 7.10(b) was taken after 4 hours when monochloramine had penetrated to about half of the initial concentration. Biofilm viability continued to decrease with time. After 24 hours disinfection, the biofilm viability was seen to decrease continuously with time (Fig. 7.10(c)). However, some live (green-colored) cells still existed on the surface of the biofilm, even though monochloramine fully penetrated.
In the presence of phosphate (1 mg P/L), monochloramine penetrated the biofilm with a similar trend as with the experiment without phosphate. The aerobic activity of the biofilm also decreased as the monochloramine penetrated, and DO began to penetrate the biofilm as well. However, DO consumption (52%) still existed at around 10 hours, retarding the DO full penetration of DO (Fig. 7.8(b)). At 10 hours, even though monochloramine was assumed to fully penetrate, based on its penetration trend in the former experiment (Fig. 7.7 (a)), it seems that monochloramine penetration did not equate to an equivalent loss in the aerobic microbial activity. Phosphate did not affect the monochloramine penetration. However, it is possible that phosphate may interfere with monochloramine disinfection by retarding the cessation of microbial activity for a while.

Figure 7.11 shows the progression of the biofilm during the monochloramine disinfection in the presence of phosphate (1 mg P/L). Even though monochloramine penetrated with about half of its initial concentration at the bottom of the biofilm after about 3 hr (Fig. 7.8(a)), intact cells still existed; it seemed that cells were not seriously affected to this point. However, the CLSM image after 24 hour monochloramine treatment in the presence of phosphate showed the mix of membrane compromised cells and non-respiring cells as shown in Fig. 7.11(c). From the Live/Dead results in both monochloramine disinfection experiments (Fig. 7.10(c) and Fig. 7.11(c)), it was concluded that monochloramine penetration did not equal a loss in viability, indicating that bacteria with intact cell membranes also were observed even after monochloramine fully penetration.

Figure 7.9 shows the phosphate concentration microprofile changes before and after the monochloramine disinfection in the presence of phosphate (1 mg P/L). The
phosphate concentration at the surface of the biofilm (0 μm) decreased from 4.5 mg P/L to 0.85 mg P/L after disinfection, and decreased from 11.0 mg P/L to 1.3 mg P/L at the 300 μm inside the biofilm, as shown in Fig. 7.9. This phosphate concentration change from the monochloramine disinfection can be explained by the result of phosphate diffusion out of the biofilm. The phosphate concentration inside the biofilm was similar to the bulk phosphate concentration when monochloramine addition was conducted.

7.5. Conclusions

The effects of phosphate on nitrifying bacteria biofilms and monochloramine penetration within simulated model drinking water distribution systems were investigated with macro- and micro-monitoring and CLSM observations. Nitrification, when well established with biofilm thickness of 150 - 350 μm, was not affected significantly by the phosphorus concentration during the operation. However, nitrification performance was increased in the operation with high phosphate concentration (12 mg P/L). This nitrification performance was connected to the biofilm thickness, indicating that the biofilm thickness decreased with time when the phosphate was limited. Reactor 1 had less thick biofilms (150 – 350 μm) on the slides than did reactor 2 (200 – 400 μm) or reactor 3 (250 – 400 μm) during the operation periods (4 months). Therefore, in the long term, phosphate is expected to act positively on biofilm development and microbial activity (i.e. nitrification) and contribute to nitrification occurrence.

From the microprofiles, the phosphate had no impact on the microbial activity in the nitrifying biofilm in the range of 0 – 12 mg P/L during the operation periods, indicating the stratification of ammonia-oxidizing activity and nitrite-oxidizing activity in the
biofilm. Phosphate microprofiles showed that phosphate contents in the biofilm was independent on the nitrifying activity. The biofilm with a high phosphorus bulk condition (i.e. 12 mg P/L) had similar phosphate concentrations (i.e. 10.7 mg P/L) within the biofilm, whereas the biofilm in the phosphorus limited condition showed low phosphate concentrations within the biofilm. Low availability of phosphorus seemed to change biofilm structure at the biofilm surface.

Phosphate did not affect the monochloramine penetration and monochloramine fully penetrated into the nitrifying biofilm within 24 hours, both with and without phosphate in the bulk solution during the disinfection. DO also fully penetrated into the biofilm, but its penetration was retarded in the presence of phosphate for a while. Bacteria with intact cell membranes were observed even after monochloramine fully penetration, indicating monochloramine penetration did not equal a loss in viability. The phosphate concentration was decreased after the monochloramine disinfection in the presence of phosphate due to the phosphate diffusion out of the biofilm.
Figure 7.1 Schematic diagram of annular biofilm reactor operation.
(a) Microelectrode setup in flow chamber for microprofiling

(b) Microprofiling experimental setup
(c) CLSM image of representative microelectrode positioning (Z stack: 142.46\(\mu\)m).

Figure 7.2 Experimental apparatus for microprofiling and microelectrode positioning.
Table 7.1  The results of the annular biofilm reactor operation

<table>
<thead>
<tr>
<th>Influent</th>
<th>Reactor 1</th>
<th>Reactor 2</th>
<th>Reactor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase 1¹)</td>
<td>Phase 2²)</td>
<td>Phase 1</td>
</tr>
<tr>
<td>Ammonia-N (mg N/L)</td>
<td>20 - 250 ³)</td>
<td>200 (100-200)⁴)</td>
<td>20 - 250 ³)</td>
</tr>
<tr>
<td>Phosphorus (mg P/L)</td>
<td>1.2 - 15 ⁵)</td>
<td>0</td>
<td>1.2 - 15 ⁵)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bulk in the reactor</th>
<th>Reactor 1</th>
<th>Reactor 2</th>
<th>Reactor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.6 (6.8 - 8.1)</td>
<td>7.5 (7.1 - 8.0)</td>
<td>7.9 (7.4 - 8.4)</td>
</tr>
<tr>
<td>Ammonia-N (mg N/L)</td>
<td>14.8 (0 - 65.8)</td>
<td>51.9 (0.3 - 215)</td>
<td>45.3 (0.1-153.0)</td>
</tr>
<tr>
<td>Nitrite-N (mg N/L)</td>
<td>103.5 ⁶) (26 - 173)</td>
<td>40.2 (1.5-149.1)</td>
<td>71.5 ⁶) (5.0-126.3)</td>
</tr>
<tr>
<td>Nitrate-N (mg N/L)</td>
<td>62.7 ⁶) (9.0 - 90.3)</td>
<td>29.4 (6.8 - 74)</td>
<td>65.1 ⁶) (51.9-88.1)</td>
</tr>
<tr>
<td>Phosphorus (mg P/L)</td>
<td>13.4 ⁶) (9.6 - 16.3)</td>
<td>0.3 (0 - 2.8)</td>
<td>13.9 ⁶) (6.0-27.5)</td>
</tr>
</tbody>
</table>

1) Phase 1: the periods with the same condition to grow the nitrifying biofilm (4 months)
2) Phase 2: the periods after changing the phosphate concentration in the influent (reactor 1: 0 mg P/L, reactor 2: 1.0 mg P/L, reactor 3: 1.2 mg P/L) (4 months)
3) Influent ammonia-N concentration was added increasingly with time in Phase 1.
4) Influent ammonia-N concentration was occasionally adjusted to low concentration when poor nitrification was observed (i.e. < 70% of ammonia-N removal) and slowly increased to 200 mg N/L with time.
5) Influent phosphate concentration was added increasingly with time in Phase 1.
6) Measured data when the influent ammonia-N was 200 - 250 mg N/L
(a) Microprofiles in the biofilm of Reactor 1

(b) Microprofiles in the biofilm of Reactor 2
(c) Microprofiles in the biofilm of Reactor 3

Figure 7.3   Microprofiles of dissolved oxygen, ammonium, pH, nitrate, and phosphate in the nitrifying biofilm in phase 2
Figure 7.4 CLSM images of the nitrifying biofilm in reactor 1 (Z stack: 115.33μm). Green and red coloring indicates live and dead cells, respectively.
Figure 7.5  CLSM images of the nitrifying biofilm in reactor 2 (Z stack: 162.81μm). Green and red coloring indicates live and dead cells, respectively.
Figure 7.6  CLSM images of the nitrifying biofilm in reactor 3 (Z stack: 162.81µm). Green and red coloring indicates live and dead cells, respectively.
Figure 7.7  Microprofiles of monochloramine and DO during the monochloramine disinfection in the absence of phosphate (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 4.6 mg Cl₂/L monochloramine)
Figure 7.8 Microprofiles of monochloramine and DO during the monochloramine disinfection in the presence of phosphate (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, 4.6 mg Cl₂/L monochloramine, and 1.0 mg P/L)
Figure 7.9  Phosphate microprofiles before and after the monochloramine disinfection in the presence of phosphate (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, 4.6 mg Cl₂/L monochloramine, and 1.0 mg P/L)
(a) Intact biofilm (before disinfection) (Z stack: 203.5µm)

(b) 4 hour after monochloramine disinfection (Z stack: 67.8µm)
Figure 7.10  Progression CLSM images of the biofilm during the monochloramine disinfection in the absence of phosphate (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, and 4.4 mg Cl₂/L monochloramine). Green and red coloring indicates live and dead cells, respectively.
(a) Intact biofilm (before disinfection)

(b) 3 hour after monochloramine disinfection
Figure 7.11  Progression CLSM images of the biofilm during the monochloramine disinfection in the presence of phosphate (5 mM buffer solution, pH 8.0, 23°C, 8.3 mg/L DO, 4.6 mg Cl₂/L monochloramine, and 1.0 mg P/L). Green and red coloring indicates live and dead cells, respectively.
Chapter 8. Conclusions and Future Research

8.1. Conclusions

The primary objective of this research was to develop, fabricate and evaluate microelectrodes to assess nitrification within chloraminated drinking water system biofilms, and to determine the effects of phosphate as a corrosion inhibitor on nitrifying bacteria biofilm. Chlorine microelectrodes for measuring monochloramine and phosphate microelectrodes for detecting phosphate ions in biological samples (i.e. biofilms, aggregates) were developed, characterized and applied for in-situ environmental analyses.

The results of this research provide an improved insight into the relationship between phosphate, used as a corrosion inhibitor, and nitrifying biofilm in chloraminated drinking water distribution systems, and a better understanding of the impact of disinfectant (i.e. chlorine, monochloramine) penetration into biofilms on microbial activity changes (i.e. DO, ammonia, nitrate, and pH microprofiles). The correlated viability upon administration of chlorine or monochloramine disinfectant allows for better prevention and control strategies for nitrification episodes in the presence of phosphate, including biofilm control.

Conclusions from this research are summarized as follows:

1. Fabrication and Evaluation of Phosphate Microelectrode for In-Situ Environmental Analysis

The microelectrode showed excellent selectivity for the orthophosphate ions ($\text{HPO}_4^{2-}$, $\text{H}_2\text{PO}_4^-$) under various environmental conditions. Alkalinity and DO were found to
interfere with the electrode’s response to phosphate. The phosphate microelectrode was also evaluated with Scanning Electron Microscopy (SEM) and Cyclic Voltammetry (CV). The developed microelectrode was used for in situ monitoring of phosphate in microbial flocs. Well-defined phosphate concentration microprofiles were measured within activated sludge flocs in the enhanced biological phosphate removal (EBPR) process, and a fluorescent in situ hybridization (FISH) and clone library analysis was also conducted to identify poly-phosphate accumulating organisms (PAOs). The center of the flocs had the highest phosphate concentrations, and the stratification of the flocs found by microprofiling indicated that the PAOs were probably distributed evenly throughout the flocs. Under the assumption that the phosphate, which was generated because of phosphate release by microbial activity, was not consumed by microbes and was only transferred from the flocs to the bulk by diffusion during anaerobic conditions, the effective diffusion coefficient (D_f) for phosphate release within the flocs was calculated to be $3.33 \times 10^{-7}$ cm$^2$/s at the end of the anaerobic phase of the EBPR process. This full characterization and successful application showed that the cobalt-based phosphate microelectrodes can be a very useful tool for in situ measurement of phosphate in various environmental conditions, including within microbial flocs.

2. Fabrication and Evaluation of Chlorine Microelectrode for Measuring Monochloramine within Drinking Water Distribution System Biofilms

Where monochloramine dominates the system, at pH 8.0 and 4:1 Cl$_2$:N, the chlorine microelectrode had good sensitivity toward monochloramine concentrations (0.1 to 30.0 mg Cl$_2$/L) at an applied potential of +550 mV (Ag/AgCl reference electrode) at 23°C, and
it was independent of pH interference at typical drinking water pH values (6-9). A linear relationship existed with monochloramine concentration for a given dissolved oxygen (DO) concentration, even at high DO concentrations. However, a 3-D (response vs. DO vs. monochloramine concentration) calibration curve was constructed because the electrode response shifted with DO changes. The chlorine microelectrode successfully measured monochloramine concentration microprofiles with high spatial resolution within a biofilm. Ultimately, this research will lead to the in-situ application of chlorine microelectrodes for measurement of monochloramine within a chloraminated drinking water system biofilm and, along with other microelectrodes (e.g., ammonia, pH, DO), will aid in elucidating nitrification phenomena occurring in the biofilm.

3. Investigation into Monochloramine Biofilm Penetration: Penetration and its Effect on Biofilm Activity and Viability

Monochloramine and DO penetration within a nitrifying biofilm were successfully profiled, showing the reduction of aerobic activity via DO penetration following application of monochloramine. Monochloramine penetrated fully into the nitrifying biofilm within 24 hours when fed at a 4:1 Cl₂:N ratio. However, the presence of excess ammonia appeared to prevent microbial deactivation. When monochloramine fully penetrated, bacteria were not all dead but some were simply inactive. It was concluded that monochloramine penetration did not necessarily equal a loss in viability, based on the Live/Dead results. Biofilm recovery occurred when disinfection stopped. Recovery was increased when excess ammonia was present during disinfection. This research
provides more understanding of the relationship between lower reactive biocide (i.e. monochloramine) penetration and microbial activity during disinfection.

4. Comparison of Disinfectant Penetration and Reaction-Diffusion Interaction between Monochloramine and Chlorine in Water Distribution Systems Biofilms

Monochloramine showed greater penetration compared to chlorine, indicating the apparent cessation of aerobic activity within the nitrifying biofilm upon the administration of chloramine disinfectant. Monochloramine penetrated into the biofilm surface layer 49 times faster than chlorine within the nitrifying biofilm and 39 times faster in the multi-species biofilm than did chlorine. On the other hand, free chlorine seems to be consumed by oxidizing the organic matter in path of the biofilm, then diffuse further, indicating very slow chlorine penetration. However, CLSM image of membrane compromised cells in biofilm after chlorination confirmed that free chlorine is much stronger than monochloramine in disinfection. Comparison of DO microprofiles, which were measured during the chlorine disinfection and monochloramine disinfection respectively, showed that the cessation of aerobic microbial activity was more complete under chloramination. The time required to attain 50% of the monochloramine biofilm penetration near the substratum was average less than 2 hours in this research. On the other hand, chlorine showed only a maximum of 9% penetration during the times when experiments were conducted, except the experiment with 9.6 mg Cl₂/L of chlorine after 24 hrs chlorine (2.6 mg Cl₂/L) disinfection; here, 35.4% chlorine penetrated into the bottom of the biofilm. The results in this study support the hypothesis (3) and (4) that monochloramine penetrates more quickly and further into the biofilm than chlorine and
monochloramine and free chlorine act differently at surfaces of biofilm composed of various bacteria, and provides useful information for use of disinfectants for biofilm control.

5. The Effects of Phosphate as a Corrosion Inhibitor on Nitrifying Biofilms and Monochloramine Penetration in Drinking Water Distribution Systems

Nitrification, when well established with biofilm thickness of 150 - 350 μm, was not affected significantly by the phosphorus concentration during the operation. However, nitrification performance was increased in the operation with high phosphate concentration (12 mg P/L). This nitrification performance was connected to the biofilm thickness, indicating that the biofilm thickness decreased with time when the phosphate was limited. Reactor 1 had less thick biofilms (150 – 350 μm) on the slides than did reactor 2 (200 – 400 μm) or reactor 3 (250 – 400 μm) during the operation periods (4 months). Therefore, in the long term, phosphate is expected to act positively on biofilm development and microbial activity (i.e. nitrification) and contribute to nitrification occurrence.

From the microprofiles, the phosphate had no impact on the microbial activity in the nitrifying biofilm in the range of 0 – 12 mg P/L during the operation periods, indicating the stratification of ammonia-oxidizing activity and nitrite-oxidizing activity in the biofilm. Phosphate microprofiles showed that phosphate contents in the biofilm was independent on the nitrifying activity. The biofilm with a high phosphorus bulk condition (i.e. 12 mg P/L) had similar phosphate concentrations (i.e. 10.7 mg P/L) within the biofilm, whereas the biofilm in the phosphorus limited condition showed low
phosphate concentrations within the biofilm. Low availability of phosphorus seemed to change biofilm structure at the biofilm surface.

Phosphate did not affect the monochloramine penetration and monochloramine fully penetrated into the nitrifying biofilm within 24 hours, both with and without phosphate in the bulk solution during the disinfection. DO also fully penetrated into the biofilm, but its penetration was retarded in the presence of phosphate for a while. Bacteria with intact cell membranes were observed even after monochloramine fully penetration, indicating monochloramine penetration did not equal a loss in viability. The phosphate concentration was decreased after the monochloramine disinfection in the presence of phosphate due to the phosphate diffusion out of the biofilm.

8.2. Proposed Future Research

The points below are proposed as possible avenues for future research:

1. **Investigation of disinfection efficacy of free chlorine at different pH:** The pH affects the HOCl: OCl\(^-\) ratio (at pH 8.0, HOCl:OCl\(^-\) ratio is about 20:80, while at pH 7.0, HOCl:OCl\(^-\) ratio is about 80:20). The literature is clear that HOCl is more effective than OCl\(^-\); therefore, it is expected that HOCl would be more reactive than OCl\(^-\). Therefore, chlorine at pH 7 is probably a worst-case penetration and pH 9 would be a best-case penetration, with pH 8 being a balance of the two.

2. **Investigation of relationship of ORP (>700mV) and disinfection (or penetration):** Preliminary experiment showed during the monochloramine disinfection that the ORP did not change much. Additional research should confirm the previous results and
compare them to chlorine disinfection. The relationship between ORP changes and
disinfectant penetration should also be explored as a biofilm control strategy.

3. **Investigation of the monochloramine penetration and microbial activities of**
**monochloramine acclimated nitrifiers within the simulated chloraminated drinking water**
**distribution system:** Preliminary experiments were performed to investigate the
possibility of acclimation of nitrifiers in the chloraminated water distribution system (0.5
- 4 mg Cl₂/L of monochloramine) over one week. Monochloramine residual was near
zero in the bulk of the system and biofilm thickness decreased due to sloughing and
detachment during the one week of operation. Nitrification also was affected by the
monochloramine injection and almost stopped. However, the CLSM analyses indicated
that bacteria in the biofilm were still alive (mostly) even though they were exposed to 0.5
- 4 mg Cl₂/L of monochloramine during that week, and after monochloramine injection
stopped, the microbial activity (i.e. nitrification) recovered within 1 - 2 weeks. These
preliminary experiment results indicate the possibility of acclimation of nitrifiers in the
chloraminated water distribution system over long periods. Once the nitrification is
achieved in the chloraminated drinking water system and monochloramine residual has
remained continuously, the monochloramine penetration and microbial activities should
be investigated with viability tests.

4. **CLSM investigation with cryosectioning:** In this research, all CLSM images were
only obtained to about half way into the biofilm because of the limitation of dye
penetration and CLSM observation depth. Therefore, biofilm beyond the ability of dye
penetration could not be observed for viability analysis. CLSM investigation into
biofilms with cryosectioning after disinfection could be used to fill the gap and confirm
the microbial activities which can be measured by microelectrodes. Investigation of spatial distribution of AOB and NOB community with fluorescent probe is also proposed.

5. Investigation of the hypothesis that “EPS control leads to biofilm control”: It is hypothesized that extracellular polymeric substances (EPS), a mixture of carbohydrate and protein organic matter typically surrounding bacteria, will considerably influence the rate of disinfectant penetration and viability. The impact of EPS during disinfection and the relationship between EPS contents (e.g. varying degrees of EPS coating) in biofilms and monochloramine penetration are recommended as future research.
References


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