IMPACT OF CHLORAMINATION ON THE DEVELOPMENT OF OLIGOTROPHIC BIOFILMS

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THESIS

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This study aimed to i) determine the effectiveness of monochloramine disinfection on biofilm control and ii) characterize the architecture and community development of laboratory-grown oligotrophic biofilms over a two-month period. Biofilm development and disinfection were realized in Center for Disease Control (CDC) reactor systems with PVC coupons as the substratum and groundwater as the seeding and growth nutrient. To compare biofilm development under disinfection against its natural development, two CDC reactors (treatment and control) were operated. In the treatment reactor, chloramination at 8.5 ± 0.2 mg Cl₂/L as combined chlorine was applied after two weeks of biofilm growth till the end of week 10. Confocal laser scanning microscopy combined with quantitative analysis using COMSTAT program revealed that disinfection resulted in a reduction of average thickness and biomass volume by 83.6% and 81.8%, respectively, and an increase in compactness by 76.5%, suggesting the formation of a thin and compact biofilm with low biomass. In contrast, biofilm development in the control reactor led to an increase in average thickness and biomass volume by a factor of 5.2 and 47.1%, and a reduction in compactness by 75.5%. As the result, thicker and fluffier biofilm architecture was observed. Biofilm community structure change was revealed by cluster analysis and non-metric multidimensional scaling based on 16S rRNA gene-based microbial fingerprinting analysis. Samples from different reactors at the same time point had a high similarity before disinfection, but became dissimilar after disinfection. This suggested that disinfection could lead to the development of a biofilm community with a distinctive community structure. Overall findings suggest that disinfection could influence the growth of multi-species biofilms on PVC surface, shape the biofilm architecture, and select a microbial community that...
can survive, adapt, or proliferate under chloramination. These findings are important to better understand biofilm growth in chloraminated drinking water distribution systems.
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1. INTRODUCTION

Drinking water distribution systems present a large part of the infrastructure of drinking water industry (Committee on Public Water Supply Distribution Systems: Assessing and Reducing Risks, National Research Council (NRC), 2006). The vast surface of distribution systems in contact with bulk liquid can support the growth of microorganisms and the formation of biofilms. These biofilms can cause instability of drinking water during distribution, and are considered as an unknown factor in risk assessment of drinking water consumption (USEPA, 2002). Understanding how microbial biofilms survive is important in understanding and predicting the performance of a distribution system, as well as better assessing risks associated with drinking water.

In United States, maintaining disinfectant residuals in distribution systems is widely used to prevent the deterioration of biological water quality. It is required by Surface Water Treatment Rule and is a best available technology for compliance with Total Coliform Rule (USEPA, 1998). However, biofilms were observed in parts of post-disinfected distribution systems including pipe sections (Ridgeway and Olson, 1981) and water meters (Hong et al., 2010). For example, in water meters, multi-species biofilms were observed (Hong et al., 2010). Thus, understanding how disinfection affects multispecies biofilm development is important.

Many studies have investigated the persistence of biofilms under disinfection, although most of them were conducted on a short time scale. Results indicate that biofilm cells were 10- to 1000- times more resistant to free chlorine disinfection than free living cells (LeChavellier et al., 1988). One of the important factors rendering the persistence of biofilms under disinfection is the diffusional resistance caused by the biofilm matrix and the ability of extracellular polymeric substances to react with or adsorb disinfectants. As a result, there is no sufficient concentration
of disinfectants to inactivate cells deep inside the biofilms (Stewart, 2003). Under disinfection over 1-2 hours, cells were shown to lose their activities in a non-uniform manner, with cells on the peripheral of biofilms losing their activities first (Huang et al., 1995). Simões and others (2010) showed that biofilms formed by different microorganisms had various levels of resistance to disinfectants, suggesting the importance of studying the response of multispecies biofilms to disinfection. However, the impact of long term exposure to disinfectants, which is more likely to happen in distributions systems, is not well understood.

The objective of this study is to determine the impact of long-term disinfection on the development of microbial biofilms in an oligotrophic environment. In specific, this study aims to investigate the development of biofilm architecture and microbial community under prolonged monochloramine disinfection in contrast to the development without disinfectants. A Center of Disease Control (CDC) biofilm reactor was adopted to develop biofilms for up to 4 - 10 weeks. Groundwater was used as the source of microbial inoculum and nutrients. Microbial biofilms were sampled at different time points during biofilm development. The community was examined with a molecular fingerprinting tool, i.e. terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997). Cells in biofilms were stained with Live/Dead staining dyes and the structure of biofilms was visualized with confocal laser scanning microscopy.
2. LITERATURE REVIEW

2.1 Biofilm-associated issues in distribution systems

Supply of safe drinking water is one of the fundamental needs of modern societies. Since early 1900s, water treatment and disinfection technologies have tremendously reduced the occurrence of waterborne diseases (Ford, 1999). While current drinking water treatment and disinfection technologies have produced safe water in treatment plants, deterioration of water quality can happen at various degrees during delivery to households. Distribution systems are identified as “the component in public water supplies to be improved for the eradication of waterborne diseases” in the coming years (NRC, 2006).

Drinking water distribution systems comprise of pipelines, valves, storage tanks, reservoirs, meters, and other components. In this vast system, water quality deterioration can arise due to multiple external and internal factors. External factors that allow microorganisms to enter the distribution systems include pipe breakage, cross connection, and backflow (NRC, 2006). Internal factors include chemical and biological processes taking place in the distributions system, and they can cause the degradation of water quality even when external intrusion is absent (NRC, 2006).

Biofilms in distribution systems can harbor intruded microorganisms during pipe leakages and become a source of internal contaminations. Biofilms can serve as a benign environment for intruded microorganisms by protecting them from disinfection in the water column. The intruded microorganisms sometimes can include pathogens, and they can be released to the water columns during biofilm detachment. This is hypothesized as “slow release mechanism” and is considered a cause for persistence of waterborne diseases long after an
intrusion event is identified and controlled (USEPA, 2002). For example, *Helicobacter pylori*, a primary pathogenic strain that causes peptic ulcers, was discovered from biofilms formed in iron mains (Park et al., 2001). At the same time, the biofilm in distribution systems is a natural source of opportunistic pathogens that can adversely impact immunocompromised populations. For example, *Legionella pneumophila* and *Mycobacterium avium* complex (MAC) were found to reside in the biofilm matrix of distribution systems (Szewyk et al., 2002). There are many other indirect ways that biofilms can cause deterioration of water quality. For instance, biofilm growth is associated with microbially-induced corrosion, which can lead to pipe breakage and compromise the physical barrier function of pipes to drinking water. Aesthetic problems such as water containing colored mats can also occur when biofilms are sloughed. Microbial products released from biofilms can consume and deplete disinfectant residual in downstream areas within a distribution system (USEPA, 2002).

2.2 Current understandings of biofilm formation

In the last two decades, the perception of microorganisms in nature has experienced a paradigm shift from viewing microorganisms as individual cells to considering microbes as communities grown in aggregates or attachments, namely biofilms (Davey and O’toole, 2000; Stoodley et al., 2002; Costerton et al., 1995). It was discovered that the majority of microorganisms in nature live in a form of microbial aggregates in structured architecture (Costerton et al., 1995). Living in biofilms provides microorganisms ecological advantages such as cooperation in metabolism and increased protection from outside environments (Davey and O’toole, 2000). The formation of monoculture, binary, and multispecies biofilms will be reviewed with the focus on functions of a structured architecture as well as the roles of microbial interaction and community development.
2.2.1 Formation of single-species biofilms

Single-species biofilms have been used as a model experiment system to elucidate the mechanism of biofilm formation. *Pseudomonas aeruginosa, P. putida, Escherichia coli,* and *Vibrio cholerae* are model microorganisms that have been extensively studied (Davey and O’toole, 2000). The initial attachment begins when bacteria sense environmental signals and change from planktonic growth to attached growth. A variety of factors including nutrients, osmolarity, pH, iron availability, oxygen tension and temperature can serve as environmental cues for this transition. Flagella, outer membrane, and pili have been proved to play important roles in the formation of *E. coli* and *V. cholerae* biofilms, although the specific functions are different. Lipopolysaccharide (LPS), an outer membrane component can assist bacterial cells to attach to a surface. Bacterial motility is known to involve in bacterial attachment and surface spreading. For example, in *Pseudomonas* biofilm formation, flagella allow bacterial cells to move to a surface, and type IV pilus is necessary for multi-layer biofilms. The formation of a mature biofilm is associated with the production of extracellular polymeric substances (EPS) (Davey and O’toole, 2000).

A stepwise developmental model has been hypothesized to explain biofilm formation (O’toole et al., 2000). This model describes biofilm formation as a well-regulated biological developmental process starting from initial attachment, the formation of microcolonies, formation of macrocolonies composed of microcolonies and water channels, to dispersal of cells from the biofilm. However, unlike other microbial developmental processes such as fruiting-body formation in *Myxococcus* (the classical model for microbial development), a hierarchical genetic network that can regulate biofilm formation has not been found. Instead, more than one
genetic pathway was observed to involve in the formation of *P. aeruginosa* and *P. putida* biofilms (Klausen et al., 2006).

One alternative hypothesis of biofilm formation describes that biofilm architecture is formed due to local ecological adaptation of microorganisms (Klausen et al., 2006). In this hypothesis, the formation of biofilms is accredited to the action of individual cells in response to environmental conditions. Surface attachment, microcolony formation, dispersal, and formation of subpopulations are regulated according to local environmental conditions. This hypothesis is supported by several experimental findings, including that local carbon starvation can trigger biofilm dissolution, and glucose and citrate can give rise to different biofilm architectures in *P. aeruginosa* (Klausen et al., 2006). Nevertheless, the complete picture of mechanisms behind biofilm formation still remains to be fully elucidated.

2.2.2 Biofilm architecture

In a mature biofilm, the architecture is characterized as cell clusters embedded in a polymeric matrix with water channels open to the environment. This architecture gives biofilms unique mass transport properties. The matrix exhibits a diffusion resistance to protons, nutrients, and oxygen. In studies on nitrifying biofilms and sulfate reducing biofilms, pH was observed to decrease along the depth in a biofilm (Okabe et al., 1999). A review on diffusion phenomena in biofilms (Stewart et al., 2003) reported that biofilms exhibit a reduced effective diffusivity at varying degrees to ammonium, nitrate, nitrous oxide, oxygen, acetate, propionate, phenol, butyrate, glucose, and sucrose. However, mass transport in water channels is significantly enhanced compared to the microcolonies. This was supported by directly monitoring the oxygen profile in a mixed culture biofilm (DeBeer et al., 1994), which showed that the oxygen concentration in a microcolony decreased sharply along the depth in a biofilm, while the
concentration of oxygen was higher in the water channel than in the microcolonies at the same depth.

In a biofilm, the activity of a bacterium was reported to associate with spatial differences. One study investigated the distribution of cell growth rate in microcolonies formed by a toluene-degrading \textit{P. putida} strain (Sternberg et al., 1999). Results indicated that cells deep inside the microcolonies had a reduced growth rate compared to cells at the peripheral of the microcolonies. The authors further tested whether nutrient availability was the reason for the difference observed, and it was observed that supplying a more easily degradable carbon source led to enhanced growth rate inside the microcolonies. The architecture of biofilms also plays an important role in protecting biofilm microorganisms from adverse environment factors and antimicrobial agents. This is further discussed in section 2.6.

2.2.3 Ecological interaction in biofilm formation

In nature, biofilms are typically composed of multiple species. To study interactions among microorganisms in biofilms in the laboratory, one methodology is to build dual species biofilms in a flow cell. Researchers use fluorescence \textit{in-situ} hybridization (FISH) to label cells according to their phylogenetic affiliations (An et al., 2005, Christensen et al., 2002), and insert gfp fusion with growth rate regulated promoter to visualize the growth activity (Christensen et al., 2002). Confocal laser scanning microscopy is used to visualize the spatial distribution of microorganisms and activity of fluorescently-labeled cells in the biofilm.

Using the aforementioned research methods, formation of microbial interspecies interactions in biofilms has been revealed. It was shown that biofilm growth enabled the formation of an interspecies relationship different from suspended growth in a chemostat for the
same strains (Christensen et al., 2002). *Acinetobacter* sp. and *P. putida* are two soil microorganisms capable of using benzyl alcohol as a sole carbon source, but *Acinetobacter* sp. has a faster growth rate with this substrate. In a chemostat growing those two microorganisms with benzyl alcohol, the interspecies interaction between these two strains appeared to be a competition relationship with *Acinetobacter* sp. outnumbering *P. putida* by 500:1. However, when growing in biofilms, *P. putida* can coexist with *Acinetobacter* sp. at a ratio of 5:1. To explain this difference, the researchers examined metabolites released and the spatial distribution of these two microorganisms in the biofilms. It was shown that benzoate, an intermediate of benzyl alcohol degradation, accumulated in the effluent of the *Acinetobacter* sp. biofilm but not in that of the dual-species biofilm, suggesting that *P. putida* could utilize benzoate but *Acinetobacter* sp. could not. During the formation of the dual-species biofilm, a spatial distribution of the two strains that can benefit the *P. putida* was observed. Initially, the biofilm was dominated by *Acinetobacter* sp. However, in the mature structure formed after a few days of co-existence, *Acinetobacter* sp. were distributed at the top of the biofilm and *P. putida* close to the substratum. This kind of structure enabled the *Acinetobacter* sp. to be close to the flow of benzyl alcohol, while at the same time gave *P. putida* the opportunity to utilize benzoate acids released from the *Acinetobacter* sp. The interspecies interaction exhibited a metabolic commensalism that was beneficial to *P. putida* and neutral to *Acinetobacter* sp.

In a dual-species biofilm study, competitive interaction between bacterial species (An et al., 2005) was investigated. The model microorganisms used in this study were *P. aeruginosa* and *Agrobacterium tumefaciens*. Competition over nutrients resulted in an overriding biomass of *P. aeruginosa* compared to *A. tumefaciens* and a spatial distribution where *P. aeruginosa* was observed to “blanket” the *A. tumefaciens* biofilm. The researchers investigated the molecular
mechanisms for this phenomenon by constructing *P. aeruginosa* mutants lacking genes regulating quorum sensing or motility. Their experiments showed that these mutants could not form “blanketing” biofilms over *A. tumefaciens*, suggesting the importance of motility and quorum sensing in the competitive advantage of *P. aeruginosa* in dual-species biofilms. Interestingly, the experiments also showed that *A. tumefaciens*, though blanketed by *P. aeruginosa*, were still viable in the dual-species biofilms, and some *A. tumefaciens* detached from the biofilm by swimming motility before they were fully blanketed. The mechanisms for their behavior in the competitive relationship still remain to be uncovered.

Biofilm formation can give microorganisms other benefits, such as sheltering from adverse environmental stress like UV exposure, desiccation, and disinfection (Davey and O’toole, 2000). For example, it has been shown that mixed-culture biofilms had higher resistance to antibiotics and bacterial invasion than monoculture biofilms, suggesting that interspecies interactions may play a role in biofilm resistance (Burmølle et al., 2006). But this phenomenon has not been well understood.

2.2.4 Community succession in multispecies biofilm formation

As biofilms in the nature are composed of multiple species, they need to be studied at a community scale. Temporal development of a microbial community is an integral part of multispecies biofilm formation and maturation. In macroecology, the temporal change of a community is viewed as the result of natural succession and influences of external factors (Odum, 1969). Community succession in multi-species biofilms has been studied in many environments, including soil biofilms (Burmolle et al., 2007), river biofilms (Jackson et al., 2001; Lyautey et al., 2005), biofilms formed on corroding concrete sewage pipes (Okabe et al., 2007), and biofilms in model drinking water distribution system (Martiny et al., 2003). It was found that community
structure and function can be altered during the succession process, and the community structure change is related to the architecture development and the spatial distribution of microorganisms in the biofilm.

A model of community succession during the development of biofilms has been proposed based on an observation of epilithic biofilms (Jackson et al., 2001). In this study, community structure was characterized with a fingerprinting tool, denaturing gradient gel electrophoresis (DGGE), and functional change was studied by monitoring the ability of the community to utilize carbon sources (i.e. glucose, cellulose, and benzoate) with different levels of biodegradability. A change in the community structure with a fluctuation in both richness and diversity was observed during the development of the biofilm. This change was associated with an increase in the community’s ability to utilize benzoate, a more difficult carbon source. To describe and possibly explain the pattern of community succession in the epilithic biofilm, the authors proposed a biofilm formation process starting with a stochastic initiation of biofilms. The community in the beginning was assumed to be random and diverse. As the biofilm developed, interactions such as competition over substrates took place and allowed some populations to become dominant at this stage. Then, a climax community with high diversity emerged. The authors interpreted that the establishment of a heterogeneous architecture was beneficial to the microorganisms in the biofilms, because a greater diversity in habitats would occur and further allow exposure to different resources. This model seems to agree with the development of a bacterial community in a model drinking water distribution system over 3 years (Martiny et al., 2003) and the development of a bacterial community in a river biofilm over 3 months (Lyautey et al., 2005).
However, in certain cases, microbial community development did not show the similar
trend as in the conceptual model discussed above, suggesting that this process can be complex
and possibly controlled by multiple factors. Studying soil biofilm development for 7 days,
Burmolle and others (2007) observed that most of the microbial populations (as resolved by
DGGE bands) were present all times, while some populations only occurred on certain days,
suggesting that microorganisms in a multispecies biofilm can include specific early, intermediate,
or late colonizers. The fluctuation in diversity postulated in Jackson’s model was not observed in
this experiment. The authors proposed that trends in community succession were related to the
phase during which the observation was conducted. While the observations on epilithic biofilms
and drinking water biofilms were conducted as long-term experiments, the study on soil biofilm
development was focused on the early succession phase. Other factors that can influence
community development were also discussed by Lyautey and coworkers (2005). They observed
that seasonal change as an allogenic factor can alter community development of river biofilms,
contrasting to biofilm development driven by natural succession.

2.3 Biofilm development in drinking water distribution systems

Biofilm development in drinking water distribution systems is an important but poorly
understood process. A few studies have looked into biofilm development in systems with and
without disinfectants (Regan et al., 2002; Santo Domingo et al., 2003; Martiny et al., 2003;
Martiny et al., 2005). Among them, the development of biofilms in a pilot distribution system
receiving Danish drinking water with low organic carbon and absence of disinfectant was well
characterized (Martiny et al., 2003; Martiny et al., 2005).

In the study by Martiny and coworkers (2003), succession in a biofilm community was
observed for over 3 years. Biofilm architecture was visualized with confocal laser scanning
microscope (CLSM), and community structure was characterized with terminal restrictive fragment polymorphism (T-RFLP) analysis and 16S rRNA gene clone libraries. After about 700 days, both biofilm community and architecture appeared to stabilize. During biofilm development, microcolonies started to be observed on day 94, and then larger microcolonies were observed. Biofilm thickness stabilized at about 300 µm on day 600. The cluster analysis for community fingerprints from T-RFLP showed that there was a clear separation of biofilms from day 1 to day 94 and from day 571 to day 1093. They categorized the biofilms from these two periods as young biofilms and old biofilms. From the 16S rRNA clone libraries constructed, twelve phyla, including Acidobacteria, Nitrospirae, Planctomycetes, and Verrucomicrobia, were detected in the system. The relative species richness, defined as the ratio of the number of operational taxonomic units over the number of sequences, fluctuated during biofilm development. In the first 14 days, the relative richness increased to 0.7, a level similar to the bulk liquid, and then dropped to 0.2 in the 256-day biofilm. The relative richness increased afterwards, and reached about 0.8 after 800 days. While the trend observed is similar to the postulated trends by Jackson et al. (2001), the authors acknowledged the possible biases associated with the measurement of relative richness.

The study by Martiny et al (2003) showed that microbial community structure could provide an indirect insight into the metabolism of biofilms in the systems and the impact of biofilms on water quality. In this study, Nitrospirae-related populations were observed to represent 25% and 39% of the sequenced 16S rRNA clones from the biofilm and bulk liquid communities, respectively. This suggested that autotrophic metabolism with nitrite as the electron donor could occur during the biofilm development. Likely, biofilm communities could play an important role in converting nitrite to nitrate in the distribution system with low levels of
organic carbons (<10 µg/L). This hypothesis was tested by a nitrite utilization experiment. It was found that biofilm samples and bulk liquid samples taken from both the inlet and outlet of the system were able to reduce nitrite levels in drinking water.

2.4 Control of biofilms in distribution systems

Control of biofilm growth is important for preventing water quality degradation in drinking water distribution system and delivering high quality water to consumers (NRC, 2006, USEPA 2002). Many different methods have been used to control biofilm growth. Limiting nutrients in the water and maintaining disinfectant residuals are two major ways of biofilm control to achieve biologically stable water in the United States of America (NRC, 2006). Frequent flushing of water mains is another way to physically remove the deposits and biofilms from pipelines (USEPA 2002). Some site-specific methods are used to tackle problems associated with biological growth in distribution systems. For example in systems that have corrosion problems, corrosion control programs such as adding corrosion inhibitors can mitigate biofilm growth. To control biofilm growth effectively, the selection of one or more methods should be made in a site specific way (USEPA, 2002). This section will discuss how nutrient limitation and maintaining disinfectant residuals affect biofilm growth.

2.4.1 Controlling biofilm growth by limiting nutrient availability in distributed water

Nutrient control is considered as one of the most effective measures in biofilm control (USEPA, 2002). Nutrients of interest include organic carbons, ammonia, and phosphorous. Limiting organic carbon has been proved to be effective in biofilm control in systems with and without disinfectant residuals (van der Kooij, 1987, Volk and LeChevallier, 1999). However, to what extent the organic carbons should be treated remains to be further determined. Assimilable organic carbon (AOC), the easily biodegradable part of organic carbon, is frequently used as the
parameter for organic carbon control. In a full-scale study in U.S. distribution systems with different levels of residual disinfectants, distribution systems that experienced coliform problems had AOC present at 100 μg C/L or higher. It was then recommended that AOC should be controlled below this threshold (LeChevallier et al., 1996). In another study by Van der Kooij (1992), 10 μg C/L of AOC was proposed as a practical limit for biologically stable water.

Besides organic carbons, ammonia and phosphorous are also important nutrients in stimulating biofilm formation in distribution systems. In water supply systems that adopt chloramines as post-disinfectants, autotrophic microorganisms can use ammonia released from monochloramine as an energy source (USEPA, 2002). Their growth metabolites can subsequently support heterotrophic growth as nutrients (Kindaichi et al., 2004). Phosphorous is generally not a growth limiting factor. However, it can be a limiting nutrient in boreal area where groundwater contains a high concentration of organic carbon. It was reported that with total organic carbon (TOC) ranging from 0.7/mg/L to 3.1mg/L, phosphate concentration of 10 μg /L can enhance microbial growth (Miettinen et al., 1997). In distribution systems where phosphorous is limited, dosing of orthophosphate-based corrosion inhibitors should be done in a cautious manner to avoid an unintentional outgrowth of biofilms (Miettinen et al., 1997).

Biological treatment can be used to remove nutrients such as organic carbon, ammonia, and nitrate from source water, and consequently reduce the nutrients entering distribution systems. One form of widely used biological treatment is river bank filtration. Such a system utilizes aquifers close to river or lake bank as the media for microbial attachment and allows degradation of organic compounds (USEPA, 2002). Another widely used technology is biological treatments with fixed-bed reactors. In such systems, biofilms developed on media such as sand and granular activated carbons can utilize the nutrients in source water (USEPA, 2002;
Brown, 2007). However, application of biological treatment in U.S. is still limited due to concerns over pathogen leaking from biologically active filters and operational challenges such as frequent clogging (Brown, 2007). Another limit with this technology is the time required to establish a functional microbial community for biological treatment. At least 3 months were required in a model distribution system to reduce biofilm growth after installation (Norton and LeChevallier, 2000).

2.4.2 Controlling biofilm growth by maintaining adequate disinfectant residuals

Maintaining disinfectant residuals is a current practice in U.S. distribution systems. It is required by Surface Water Treatment Rule and is the best available technology for compliance with Total Coliform Rule (NRC, 2006). The most widely used residual disinfectants are free chlorine, chloramines, and chlorine dioxide (Crittenden et al., 2002). Effectiveness of residual disinfectants to control biofilm growth has been supported by studies on many systems (USEPA, 2002). For example, in a study on full-scale distribution systems, systems that maintained dead-end free chlorine level \(\geq 0.2\) mg/L or chloramine \(\geq 0.5\) mg/L had less coliform growth and biofilm formation than systems that maintained less adequate disinfectant residuals (LeChevallier et al., 1996).

Although both free chlorine and chloramines can be used as disinfectant residuals, chloramines are generally preferred to achieve better biofilm growth control (Trussell, 1999). One explanation is that free chlorine is more reactive than chloramines and can react with matrix materials in biofilms, therefore it would not be able to sustain a high concentration to penetrate through the biofilm (LeChevallier et al., 1988). The advantage of using monochloramine in biofilm disinfection was shown in a laboratory study (LeChevallier et al., 1988). In this study, bacterial isolates from drinking water were used to form biofilms on metal coupons and granular
activated carbon. Chloramine was shown to take a shorter contact time (CT) to inactivate biofilm bacteria in both cases. Another consequence of the relatively lower reactivity of chloramines is that they are more stable in the distribution system. Therefore its tendency to decay is lower than free chlorine (Trussel, 1999). In another study comparing the efficacy of free and combined chlorine in controlling biofilm growth, coliform occurrence in systems using chloramines was about half of that in systems using free chlorine (distribution systems used in this study receive water from treatment process that include filters) (LeChevallier et al., 1996).

The effectiveness of disinfectants to control biofilm growth is affected by many site-specific factors. The concentration of disinfectants is important in controlling biofilm growth, therefore any reaction that consumes disinfectants will make biofilm control difficult. In distribution systems that have corrosion problems, corrosion products can react with both free chlorine and chloramine, lower their concentrations, and cause ineffective biofilm control (LeChevallier et al., 1996). pH is an important water chemistry characteristics that affects the effectiveness of disinfectants. Monochloramine, the major disinfectant in chloramines, is more stable under pH 7-8.5, and failure to maintain an adequate pH will cause the equilibrium towards less effective species (USEPA, 2002; Crittenden et al., 2002). Water temperature in a distribution system is another environmental factor that affects disinfection effectiveness, but this can hardly be controlled in distribution systems by engineering protocols (LeChevallier et al., 1996).

Although the concentration of disinfectants in distribution systems has a significant impact on biofilm control, the dosage needs to be balanced to prevent formation of disinfection byproducts (DBPs). In 1976, chloroform, a trihalomethane and DBP derived from chlorination, was found to be carcinogenic in rodents. Later studies found a weak correlation between bladder cancer and DBPs (King and Marrett, 1996). This triggers EPA to regulate disinfection practice
for a tradeoff between biological quality control and risks from exposure to DBPs (Boorman et al., 1999). DBPs formation is not only a problem associated with chlorination, but also with chloramination. N-nitrosodimethylamine (NDMA), a probable human carcinogen, was found to form in chloramination (Choi and Valentine, 2002). In EPA regulations, both DBPs and disinfectant residuals are regulated. In Stage I Disinfection/Disinfection Product Rule, the maximum detection limits of free chlorine and chloramine in distribution system are regulated as an annual average at 4 mg/L as Cl₂ (USEPA, 1998).

2.5 Impact of disinfection on microbial biofilms

Since disinfectants are routinely applied in distribution systems in the U.S., researchers have tried to assess the impact of disinfection practice on biofilm formation in distribution systems. Scenarios of importance in engineering settings were studied, such as discontinuous disinfection (Codony et al., 2005; Mathieu et al., 2009) and switching of disinfectant types (Santo Domingo et al., 2003; Pryor et al., 2004). These studies presented interesting phenomena on the response of multi-species biofilms in drinking water distribution systems to disinfection practice.

Codony and coworkers (2005) studied the impact of discontinuous chlorination on release of microbial cells into the water phase. This scenario occurs in chlorinated systems when there is a fluctuation in the chlorine demand of influent water. The researchers studied the increase in effluent cell numbers against inlet cell numbers. The results showed that total cell count and heterotrophic plate counts had different responses to discontinuous disinfection. Total cell counts were reduced by 10 fold after chlorination, and this reduction was not affected by repeated disinfection. In contrast, heterotrophic plate count exhibited a 4-log decrease in the first round of chlorination, but the rate of decrease in the number of heterotrophic bacteria was lower.
after repeated disinfection. From a microbial ecology point of view, this study showed that different groups in a drinking water community may have different levels of resistance to chlorine disinfection. It also indicates culture-based methods for enumerating microorganisms ignore certain aspects of the microbial quality of drinking water.

Another study on the effects of disinfection attempted to address the change in specific groups of microorganisms during disinfection directly on biofilms (Mathieu et al., 2009). Biofilm samples were retrieved from a pilot system experiencing fluctuating disinfectants (0.1-0.4-0.1 mg Cl₂/L). Using Live/Dead stain, the authors found that discontinuous disinfection did not cause a significant variation in total cell number and the number of cells with intact membranes, while the FISH result suggested that the relative abundances of α-, β-, γ-Proteobacteria varied. Increased level of chlorination was associated with higher relative abundance of β- and γ-Proteobacteria and a lower relative abundance of α-Proteobacteria. This change was reversed after the disinfectant level dropped, indicating the resilience of microbial community.

In recent years, there is a trend to switch from chlorination to chloramination. It is important to understand the impact of such changes in water chemistry on microbial community. Studies have been conducted to compare the community structure under different disinfectant types and a direct consequence of a change in the disinfection regime. A study conducted by EPA lab investigated the shift of heterotrophic bacteria and ammonia-oxidizing bacteria in biofilms developed in a water distribution system simulator (Santo Domingo et al., 2003). Using culture-based methods, the researchers found that monochloramine disinfection reduced the abundance of heterotrophic bacteria and increased the abundance of ammonia oxidizing bacteria. Pryor and coworkers (2004) studied the impact of disinfectant shift on the occurrence of
pathogen in a municipal drinking water system in Florida. Prior to the switch of disinfectant, *Legionella* species and *Mycobacterium* species were found in biofilms in the chlorinated distribution systems. Among the opportunistic pathogenic species, *Legionella pneumophila* was not found, while *Mycobacterium avium* and *M. intracellulare* were found. The point-of-use levels of *Legionella* and *Mycobacterium* were affected after the disinfectant switch. *Legionella* colonization rate on the shower heads were decreased from 20% to 6.2% and *L. pneumophila* remained the same. The colonization rate of *Mycobacterium* species increased from 19.1% to 42.2%. These studies suggested the change in microbial communities caused by disinfection may have important public health implications.

2.6 The resistance of biofilms to disinfectants

Biofilm bacteria have been observed to be resistant to drinking water disinfectants such as hypochlorous acid, monochloramine, and chlorine dioxide (LeChevallier, 1988). One study investigated the efficacy of disinfectants to coliform bacteria and heterotrophic plate count (HPC) bacteria growing on different surfaces, and used concentration-time product (CT) required for 99% inactivation as a parameter to compare. It was found that the CT product for biofilm inactivation is 2-100 fold higher in monochloramine disinfection and 150 to more than 3000 times higher in free chlorine disinfection.

Failure of free or combined chlorine to penetrate biofilms is considered as an important reason for biofilms to resist chlorine-based disinfection. Studies on penetration of disinfectants in biofilms adopt experimental systems of biofilms grown on coupons in annular reactors (De Beer et al., 1994, Huang et al., 1995) or artificial biofilms (Chen and Stewart, 1996). Biofilms are exposed to a bulk liquid containing known concentrations of free chlorine or monochloramine. In order to measure the concentrations of certain disinfectants like hypochlorous acid and
monochloramine along different depths in the biofilms, microelectrodes are used (De Beer et al., 1994; Lu, 2008). The tips of microelectrodes are typically 1-10 μm, making them possible to take measurements without destructing the biofilms. In such studies, a time scale of hours was typically used (De Beer et al., 1994, Huang et al., 1995, Lee et al., 2010), and biofilms were grown in nutrient rich medium to obtain thick biofilms which allows better reproducibility (Lee et al., 2010).

Direct evidence of free chlorine decay during biofilm penetration was shown using microelectrodes in a dual-species biofilm (De Beer et al., 1994). In this study, a binary biofilm of \textit{P. aeruginosa} and \textit{Klebsiella pneumoniae} with a maximum thickness of 150 to 200 μm was produced. Free chlorine concentrations ranging from 0.07-0.42 mM were tested under an exposure time of 3-105 min. This study showed an incomplete penetration of free chlorine at varying bulk liquid free chlorine concentrations and exposure time. A concentration of as low as 20% of the bulk liquid concentration was detected at the bottom of the biofilm. In a following study, an unsteady reaction-diffusion model was proposed for chlorine penetration and reaction (Chen and Stewart, 1996). Chlorine penetration was retarded by cell biomass, and the retardation was proportional to the amount of cells originally present in the biofilm. The model was tested in an artificial biofilm made of cells embedded in agarose and the experiment was in agreement with the model prediction.

Monochloramine penetration into biofilms has been characterized recently. By controlling the thickness of biofilms, the penetration was observed to be dependent on bulk liquid concentration, exposure time, and materials of substratum. In a biofilm with PVC as the substratum, monochloramine penetration can reach complete penetration within 15 min when bulk liquid concentration was 6.6 mg Cl\textsubscript{2}/L. Concrete substratum was shown to be able to react
with monochloramine, and caused a decrease in monochloramine concentration in the biofilm close to the substratum. The monochloramine concentration profile changed as exposure time extended. As exposure time increased, the concentration of monochloramine at both biofilm surface and substratum increased (Lu, 2008).

In short-term disinfection studies, the response of biofilms has been characterized to certain levels. In some studies, residual biofilm cells were scraped off from the substratum, and measured by plate counting (LeChevallier et al., 1988; Xu et al., 1995), direct counting with nucleic acid stain (Stewart et al., 1994), or redox dye staining such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Stewart et al., 1994; Huang et al., 1995). Results were reported as CFU or number of cells per substratum area. It was shown that CTC-reducing cells and total direct cell counts were in higher number than R2A agar plate colony counts (Stewart et al., 1994), suggesting the importance of using culture-independent, in-situ observation methods to characterize the response of biofilms to disinfection.

In order to reveal the spatial information in biofilms, cryosectioned-biofilms were stained with redox dye (CTC) and nucleic acid dye (4',6-diamidino-2-phenylindole) to determine active and total cell numbers (Huang et al., 1995, Stewart et al., 1994). The loss of respiratory activities was found to be non-uniform. The interface of biofilm and bulk liquid had a greater loss of respiratory activity, whereas cells close to the substratum and at the center of clusters had the highest residual respiratory activity (Huang et al., 1995). It would be logical to reason that biofilm response to disinfection would be different as experimental period extends. However the effect of long-term disinfection remains poorly understood.
Although biofilms in nature are mostly composed of multiple species, current understanding of the relationship between the ecological properties of a biofilm community and its response to disinfection is still limited. A recent study showed that diversity of biofilms can influence the resistance to free chlorine disinfection (Simões et al., 2010). In this study, six strains were isolated from a model drinking water distribution system and used to form single species biofilms, biofilms composed of five strains, or biofilms composed of all six strains. Disinfection was conducted with hypochlorous acid disinfection at concentrations ranging from 0.1 mg/L as Cl₂ to 10 mg/L as Cl₂ for 1 hour. The single species biofilms were different in their ability to survive under free chlorine disinfection. The biofilm formed by *Methylobacterium* sp. was the most resistant and biofilm formed by *Acinetobacter calcoaceticus* was the most susceptible. The mixed biofilm containing all six strains was more resistant towards disinfectants than biofilms comprising of only a single species, suggesting that multispecies biofilms may have a better ability to persist under disinfection. The resistance of biofilms composed of five strains was observed to be related to its community composition. The limit of this study is that experiments were performed in a short time scale and the community in the residual biofilms was not characterized. Still, much remains to be explored in the response of multispecies biofilms to disinfection in a time scale similar to real-world practice.
3. MATERIALS AND METHODS

3.1 Experiment design

Two series of experiments were conducted in this study (Figure 2). In both experiments, biofilm development and disinfection were achieved in Center of Disease Control biofilm reactors (Goeres et al., 2005). Two reactors were used in each experiment. The reactors were first fed with groundwater to allow initial biofilm growth for two weeks, and then subjected to monochloramine treatment (treatment reactor) or allowed to undergo natural development (control reactor). In the first run, chloramine disinfection lasted 4 weeks. Biofilms were sampled from the coupons for community analysis before disinfection, and after 2 days, 1 week, and 2 weeks of disinfection. The second run had an 8-week disinfection time. Biofilms were sampled for community analysis before disinfection, and after 4 and 8 weeks of disinfection. Fresh biofilm samples were taken also for microscopic examination and image analysis.

3.2 Reactor operation

3.2.1 Reactor system

Figure 3 shows the schematic diagram of a system for the development of biofilms with and without the treatment of disinfection. The setup included a Center for Disease Control (CDC) reactor (Biosurface technologies, MT), a 10-L carboy as a reservoir, a digital magnetic stirring plate, and a peristaltic pump (Figure 3 A). The CDC reactor with a working volume of 300 mL contains eight rods inserted (Figure 3 B). Each rod can hold three PVC coupons (Figure 3 C) to provide a surface area for bacterial attachment. A baffled stirrer was placed at the bottom of the reactor, and was controlled by a digital magnetic stirrer at 125 revolution per minute (rpm) to create a consistent fluidic shear across all the coupons. The groundwater in this study was collected from Newmark Civil Engineering Lab (205 N Mathews Avenue, Urbana, IL 61801),
and pretreated with greensand filter to remove manganese and iron. To develop multispecies biofilms, the CDC reactor and the carboy were first autoclaved, and then filled with the groundwater as seeding and growth nutrient. The reactor was operated with a hydraulic retention time of 4 hr. During the operation, the system was completely covered with aluminum foil to prevent phototrophic bacteria growth.

3.2.2 Chloramination

Monochloramine treatment to biofilms was conducted in a pulse-step mode. Groundwater was chloraminated, filled into the reactor, and then supplied at a steady flow rate. The chloraminated groundwater was prepared by adding 35 mL of freshly-prepared monochloramine concentrated solution (weight ratio of chlorine and ammonia was 5:1) into every liter of groundwater at a targeted combined chlorine concentration of 10 mg Cl₂/L. Concentration of total and free chlorine was measured with an \( N,N \)-diethyl-p-phenylenediamine (DPD) chlorine test kit (Hach, CO). The free chlorine and total chlorine concentrations in Newmark groundwater were zero.

3.3 Water quality analysis

Non-volatile organic carbon was measured with a Tekmar Dohrmann Apollo 9000 HS carbon analyzer based on Standard Method 5310B. Metals analyses were performed using a Varian Vista Pro CCD simultaneous inductively coupled plasma optical emission spectrometer based on US EPA Method 200.7. Nitrate and sulfate were analyzed with Dionex DX-500 and ICS-5000 ion chromatographs based on US EPA Method 300.0. Nitrite and ammonia analyses were performed using automated colorimetry based on US EPA Method 353.2 and 350.1, respectively. Orthophosphate analysis was performed using automated colorimetry based on US
EPA Method 365.1 and 365.4 respectively. pH and DO were measured with Orion 4 star portable pH DO meter.

3.4 Biomass retrieval and preservation

As described in section 3.1, biofilms from those two experiments at several time points were retrieved for further analysis. To do so, coupons were transferred to sterile 1X PBS buffer and physically scraped with sterile cotton swabs. The cotton swabs were washed with the same buffer 3 times by vortexing. Biomass in the buffer solution was pelleted by centrifugation at 10,000 g. For simultaneous extraction of community DNA and RNA, the samples were treated with RNAlater ICE (Ambion, TX) and processed in the same solution. Suspended biomass samples were obtained before disinfection and at the end of each experiment by filtering all liquid in the reactor (300 mL) through 0.22 μm polycarbonate filters (Millipore, MA). Groundwater biomass was collected by filtering the groundwater through a 0.22 μm polycarbonate filter at two different time points over the experiment period. Samples were preserved at -80°C before DNA extraction.

3.5 DNA extraction

Total community DNA was extracted according to a published protocol (Schmidt, et al., 1991) with some modifications. The cell pellets were re-suspended in 600 μL extraction buffer containing 0.1 M Tris-HCl, 0.1 M EDTA and 0.75 M sucrose. The mixture was transferred to a centrifuge tube containing glass beads, and was treated with 4 cycles of 30-s bead beating and 30-s ice cooling. The samples were enzymatically lyzed by lysozyme and achromopeptidase at a final concentration of 5 mg/mL and 10 μg/mL, respectively. The mixture was incubated at 37 °C for 30 min with shaking horizontally at around 225 rpm. After that, proteinase K and sodium dodecyl sulfate at a final concentration of 0.1 mg/mL and 1% wt/vol, respectively, were added to
the reaction. The mixture was further incubated at 37 °C for 2 hr with horizontal shaking at 225 rpm. Then, 50 μL of 10% hexadecyltrimethyl ammonium bromide (CTAB) and 84 μL of 5 M sodium chloride were added to the mixture. After incubation at 60 °C for 30 min, the mixture was extracted with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) twice, and equal volumes of chloroform-isoamyl alcohol (24:1) twice. The supernatant was precipitated with an equal volume of isopropanol at -20°C overnight and centrifuged at 12,000 rpm for 5 min. The pellet was carefully washed with 1 mL 70% ethanol twice. The DNA was air dried and re-dissolved with 50 μL milli-Q water. The amount of extraction products was measured with Nanodrop (Thermo scientific, DE). DNA samples were stored at -80°C prior to PCR.

Samples from the 10-week experiment were subjected to the same cell disruption and lysis. To extract total nucleic acid and fractionate DNA and RNA, the lysed mixture was washed with chloroform:IAA (24:1) by continuously inverting for 5 min and centrifuged at 7500 rpm for 10 min. The aqueous layer was collected and transferred to a 1.5 mL centrifuge tube. The reaction was added with 0.6 volume of isopropanol, mixed gently, incubated at room temperature for 30 min, and centrifuged at 10,000 rpm for 30 min. The pellet was washed with 70% ethanol twice, air dried, and re-dissolved in 50 μL of RNase free water. The product was examined on a freshly prepared 0.8% agarose gel through electrophoresis. Fractionation was conducted with QIAGEN AllPrep DNA/RNA mini kit (Qiagen, CA) according to manufacturer’s instruction. DNA and RNA samples were stored separately at -80°C.

3.6 T-RFLP

T-RFLP was conducted according to a protocol published previously (Liu et al., 1997). 16S rRNA gene was amplified with primer set 47F and 927R (Table 1). The forward primer was labeled with 6-FAM. PCR reactions were conducted in a Bio-rad C1000 thermal cycler (Bio-rad,
CA). The reaction (25 μL) contains 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.025 U/μL Taq polymerase, 1 μM forward primer, 1 μM reverse primer, and 10 ng of extracted DNA as template. The reaction condition included an initial denaturation (95 °C for 3 min), 25 cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s), and extension (72 °C for 1.5 min), and a final extension (72°C for 1.5 min). Each reaction product was examined by gel electrophoresis with 1.0% agarose gel in 1x TAE buffer at 100V for 30 min. For PCR reactions with cDNA templates, a RT-minus control was conducted. Two PCR reactions were conducted for each template and combined in one PCR tube. To remove single-stranded DNA that would lead to the formation of pseudo-restriction fragments in the coming steps, PCR products were digested with mungbean enzyme (New England Biolabs, MA) (Egert and Michael, 2003). The products were then purified with Promega Wizard SV gel and PCR clean-up system (Promega, WI), digested with tetrameric restrictive enzymes (New England Biolabs, MA) at 37 °C overnight with a final restrictive enzyme activity of 2.0 U/μL, and incubated at 65 °C for 20 min to inactivate the restrictive enzymes. The restrictive enzymes used in this study were HhaI and MspI. The digestion products were analyzed with ABI 3730 XL genetic analyzer at Roy J. Carver Biotechnology center at the University of Illinois.

T-RFLP results were analyzed with Genemapper V 4.0, and peak binning was conducted with T-RFLPstat program based on R program (Abdo et al., 2009). Statistical analysis was performed with PRIMER 6 software (Plymouth Marine Laboratory, UK). A distance matrix based on Bray-Curtis distance between samples was generated. The data were presented by cluster analysis and non-metric multidimensional scaling.
3.7 Microscopy and image analysis of biofilm architecture

Bacterial cells in the biofilm were stained with LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen, CA) according to manufacturer’s instruction. The fluorescence stains in this kit are SYTO 9 and propidium iodide (PI). According to the manual, SYTO 9 stains all cells and PI only penetrates cells with damaged cell membrane. Fresh biofilm samples were stained by adding 20 μL of working solution onto the biofilm and incubating in dark for 30 min. Stained biofilm samples were visualized with an LSM 710 confocal laser scanning microscope (Zeiss, Germany). The SYTO 9 signals were scanned by a 488-nm laser, and the PI signals were scanned by a 581-nm laser. Four vertical scans were performed to increase the signal-to-noise ratio. Seven images were obtained for each sample at each time point.

For image analysis, extended –focus images and vertical cross sections through the biofilm were generated using the IMARIS (Bitplane, Switzerland) software package. Quantitative analysis of the images was performed with COMSTAT software package (Heydorn, 2000). In the program setting, biomass signals were defined as all connected regions above a defined volume. The same thresholds for each channel were used throughout the experiments. Parameters of biovolume (μm$^3$ per μm$^2$ area), average thickness (μm), and maximum thickness (μm) were calculated based on SYTO 9 signals. The ratio between biovolume results in SYTO 9 and PI channels were further calculated to indicate the ratio of total cells and dead cells.
4. RESULTS

4.1 Groundwater quality

Table 2 summarizes water chemistry data of Newmark groundwater measured at three different times. Total organic carbon (TOC) as non-volatile organic carbon which was the sole carbon source for microbial growth had an average value of 4.03 mg/L. It was comparable to drinking water TOC levels, typically ranging from 0.05 mg/L to 12.2 mg/L (n=80) in the United States (LeChevallier et al., 1991). The groundwater contained 0.91 mg NH3-N/L, low in nitrite (0.054 mgNO2\(^{-}\)-N/L), nitrate (<0.07 mgNO3\(^{-}\)-N/L), and orthophosphate (0.029 mg-P/L).

4.2 Selection of restriction enzymes in T-RFLP analysis

In T-RFLP analysis, an inappropriate selection of restrictive enzymes may cause underestimation of community diversity and therefore renders difficulty in comparison of sample subjected to different treatment. Restrictive enzymes commonly used in T-RFLP analysis include for example MspI, HhaI, HaeIII, and AluI. In this study, two restrictive enzymes MspI and HhaI were tested on their ability to resolve groundwater biofilm samples before and after disinfection. A short-term chloramination was conducted with the CDC reactor after 2 weeks of biofilm growth. Samples were taken before disinfection, 24 hours after disinfection, and 48 hours after disinfection. With MspI digestion, the numbers of resolved peaks from the three samples were 15, 18, and 13, whereas with HhaI digestion, the numbers of resolved peaks in corresponding samples were 3, 8, and 9 (Figure 4). This result suggested that for biofilm samples taken before and after disinfection, MspI was able to resolve more peaks in T-RFLP analysis compared to HhaI. At the same time, the dominant peaks in MspI digestion products had a lower relative
abundance compared to *HhaI* digestion products. Therefore in the following long term experiments, *MspI* was used as the restriction enzyme in the T-RFLP analysis.

4.3 Impact of monochloramine disinfection on groundwater biofilm community structure- a 4-week experiment

4.3.1 Disinfection operation and biofilm sample collection

Figure 5 illustrates the actual concentration of the disinfectant in the reactor influent over the disinfection period. Arrows indicate the time points where biofilm samples were collected. Influent disinfectant levels were reported as the average concentration in each batch of prepared disinfectants. The average total chlorine concentration during the 2-week disinfection was 10.2 ± 1.2 mg Cl_2/L (average ± standard deviation). There was a low reading of free chlorine (less than 5%), likely due to the chemical equilibrium of monochloramine and free chlorine, or the errors with the DPD method used for chlorine measurements. Although the dosing of disinfectants was identical each time the disinfectant was refilled, the source water contained chlorine demand that could vary with time and this resulted in the fluctuation of the actual disinfectant level.

4.3.2 Community analysis

Figure 6 indicates the T-RFLP profiles of microbial communities from biofilm samples in both reactors. It was observed that the community structure in biofilm samples with and without disinfection shared a lot of similarity. Some peaks, including fragment lengths of 106 bp, 361 bp, 363 bp, 398 bp, 447 bp, and 456 bp, were present in all the samples. These peaks occupied 20% of all the resolved peaks. Among these peaks, the 106-bp fragment had a relative abundance over 50% in all the samples. The changes in relative abundance of some major peaks suggested changes in community structure during biofilm development. For example, 99-bp fragment was
observed only in the 2-week old biofilm sample in the control reactor. This suggested that some bacterial groups were dominant only at the early colonization stage, but their abundance dropped as the biofilm developed. Likewise, the 104-bp fragment in the control reactor was only resolved in the 4-week old biofilm, suggesting some bacterial groups were rising as the biofilm aged. In the treatment reactor, the 450-bp fragments were not detected before disinfection and 48 hours after disinfection, but were detected from samples taken one or two weeks after disinfection. This suggests that some microorganisms were not abundant in the biofilm before disinfection, but became more abundant after disinfection. Possibly, these populations were more resistant to disinfectants or were protected from disinfection.

To compare the similarity and differences of the community structure from different samples, exploratory tools including cluster analysis (Figure 7) and non-metric multidimensional analysis (MDS) (Figure 8) were used. Both relative abundance of T-RFs (Figure 7, panel A and Figure 8, panel A) and presence/absence of T-RFs (Figure 7, panel B and Figure 8, panel B) were used to calculate similarity matrix. Trends associated with treatment were not found. The observation suggested that experiment with a longer period of chloramination was necessary to detect possible changes in microbial community structure.

4.4 Impact of monochloramine disinfection on groundwater biofilm architecture and community-a 10-week experiment

4.4.1 Disinfection operation and biofilm sample collection

Figure 9 presents disinfectant concentration in treatment reactor in the second run of experiment. Chloramines disinfection was started 2 weeks after biofilm incubation and was continued for 8 more weeks. The disinfection manner was the same as in the 4-week experiment. The total chlorine level in the influent and the reactor was $11.3 \pm 1.2$ mg Cl$_2$/L and $9.1 \pm 1.0$ mg Cl$_2$/L.
Cl₂/L, respectively. Trace amount of free chlorine was detected in the chloraminated groundwater (7.0% and 6.6% of total chlorine in reactor influent and bulk liquid, respectively). The combined chlorine, or the difference between total chlorine and free chlorine was 8.5 ± 0.2 mg Cl₂/L.

In the 10-week experiment, the pH and DO in the reactors were monitored (Figure 10). pH in the control and the treatment reactor were 8.2±0.2 and 8.3±0.2, respectively. The DO concentrations in both reactors were also similar with an average value of 8.17±0.46 mg/L in the control reactor and 8.22±0.35 mg/L in the treatment reactor.

4.4.2 Development of biofilm architecture in the absence of disinfectants

Figure 11 illustrates the development of biofilm structure in the control reactor. Both extended-focus pictures (Figure 11 A, C, and E) and 3-D reconstruction pictures (Figure 11 B, D, and F) were shown. The green signals indicated SYTO-9 stained cells or total cells, while the red signals indicated PI stained cells or membrane-damaged cells. Overall, the biofilm was developed towards a more complex architecture during the 10 weeks. In the 2-week old samples (Figure 11 A and B), biofilm architecture was flat and thin. In the 6-week old biofilm samples (Figure 11 C and D), several clusters of aggregated cells (indicated with arrows in Figure 11 C) were observed with lot of space not occupied by cells. At week 10, the biofilm became thicker with a more complex structure. A lot of cell clusters were observed as indicated with arrows in the extended focus image from 10-week old biofilms (Figure 10 E). The 10-week old biofilm also exhibited a lot of void space (Figure 10 F), which may represent channels in the biofilm. Higher numbers of red signals occurred in the 6-week and 10-week old biofilm images, suggesting that the number of dead cells increased as the biofilm aged. Pictures were also taken under a reflected light mode for 10-week old biofilm samples (Figure 12). In these images, the
biofilm matrix that was not stained by fluorescent dyes can be visualized. Large space of the biofilm was not stained with fluorescent signals or did not contain matrix materials. This space is likely to represent channel and pore structures that are important for mass transport in biofilms. The reflected-light images also show that some cells close to the substratum could not be visualized with fluorescent dyes.

4.4.3 Development of biofilm architecture under chloramination

Development of biofilm architecture under chloramination was different from the control reactor. The biofilm had a flat and compact structure with cells in clusters. Reflected-light images show that the cells were embedded in biofilm matrix (Figure 14). It was observed in the 6-week old biofilm that signals from the cells were very weak and could not be properly visualized under the same conditions as two-week old biofilms in CLSM. In order to obtain properly fluorescent images, 10-week old biofilm samples in the treatment reactor were visualized with compensated laser power. Three un-compensated image sets were also taken in order to provide a basis to compare the pictures taken for 6-week old biofilms against 10-week old biofilms (images not shown).

4.4.4 Quantitative analysis of biofilm architecture

Quantitative analysis of biofilm image data were used to compare the development of biofilm architecture with and without of monochloramine disinfection. Biomass volume, average thickness of biofilms, and maximum thickness of biofilms were selected as the parameters to compare the differences between the biofilm structures. As the 6-week old biofilm samples from the treatment reactor had a lower fluorescence under confocal laser scanning microscope, laser compensation was applied to the 10-week old biofilm to obtain adequate images. Non-compensated images were also obtained for the purpose to compare between non-compensated
and compensated images. Overall, development of biofilm structure was significantly affected by disinfection. At week 2 (no disinfection applied), the architecture of biofilms from control reactor and the treatment reactor were not significantly different (ANOVA, \( p > 0.05 \) for biomass volume, average thickness, and maximum thickness). At weeks 6 and 10, biofilm architectures from the two reactors were significantly different (ANOVA, \( p < 0.05 \) for biomass volume, average thickness, and maximum thickness).

Increase in biomass volume was observed during biofilm development in the control reactor (Figure 15, A and C) but not in the treatment reactor (Figure 15, B and D). Biofilms with disinfection throughout the experiment had a reduction in average thickness and biomass volume by 83.6\% (Figure 15 A) and 81.8\% (Figure 15 C), respectively, whereas natural development in the control reactor led to an increase in average thickness and biomass volume by 519\% (Figure 15 B) and 47.1\% (Figure 15 D), respectively. Maximum thickness increased with biofilms in the control reactor but not the treatment reactor (Figure 15 E and F). It is possible to calculate the ratio of biomass volume and average thickness and represent the volume of cells in unit volume of biofilms. This ratio can indicate the compactness of the biofilm (Equation i). Figure 16 shows that the compactness of biofilm increased by 76.5\% with disinfection and dropped by 75.5\% without disinfection.

\[
\text{Compactness} = \frac{\text{biomass volume}}{\text{biofilm volume}} = \frac{\text{biovolume per unit area} \times \text{biofilm area}}{\text{biofilm mean thickness} \times \text{biofilm area}} = \frac{\text{biovolume}}{\text{biofilm mean thickness}} \quad (i)
\]

Table 3 summarizes the ratios of biomass volume in green and red signals of live/dead BacLight staining. In both reactors, the ratio dropped as the biofilm developed, suggesting that dead cells increased as biofilms aged. In the treatment reactor, live cells were still present 4 weeks or 8 weeks after disinfection, and the green/red ratio was comparable to the old biofilm.
samples from control reactor. This indicated some microorganisms could survive after disinfection treatment.

4.4.5 Microbial community analysis of biofilm samples taken from the 10-week experiment

Figure 17 indicates the T-RFLP fingerprints of biofilm samples retrieved in the 10-week experiment are presented in Figure 17. Among bulk liquid samples, only the 2-week old sample from the treatment reactor (before disinfection started) were successfully analyzed with T-RFLP (data included in statistical analysis in 4.4.6). T-RFLP fingerprints of biofilm samples from the control reactor show that the biofilm community changed during its development. Two-week (Figure 17 A), 6-week (Figure 17 C), and 10-week old (Figure 17E) biofilms shared some common peaks, (e.g., 106-bp and 404-bp fragments), but differed in their relative abundance. The early biofilm sample (Figure 17 A) was characterized by the dominance of two major peaks, 106-bp and 404-bp T-RFs, while 6-week old and 10-week old biofilm samples contained many minor peaks at low abundance (panel C and E).

Before disinfection was applied, biofilm communities from the control and treatment reactors were similar to each other (Figure 17 B). Ten of the 14 peaks observed in the 2-week old sample were shared, and two dominant peaks, 106-bp (36.8%) and 404-bp (25.4%) were the same. The fingerprints for biofilm samples after disinfection (Figure 17 D and F) were quite different. The 404-bp fragment, dominant in the 2-week old biofilms, was present at low abundance in the 6-week old biofilm sample (1.7%), and was not detected in the 10-week old biofilm sample. Instead, 236-bp fragment became one of the dominant peaks after disinfection. In the control reactor, increase in the abundance of 236-bp fragment was not observed. Instead, 88-bp and 400-bp became more abundant as the biofilm aged.
Comparing the peaks across reactors, some of the peaks would represent groups of microorganisms that are important in understanding biofilm development under chloramination. Four peaks (106-bp, 363-bp, 404-bp, and 450-bp) were shared by all the biofilm samples, possibly represent microorganisms that are important in the formation of biofilms during the experiment period. The presence of certain peaks only in disinfected samples as mentioned above may imply that some microorganisms were more adapted under chloramination.

4.4.6 Statistical analysis of T-RFLP data

Cluster analysis was applied to the T-RFLP data with a bulk liquid sample included as a positive control. The result without transformation (Figure 18 A) indicates that the two-week old biofilm samples from different reactors were clustered together with the bulk liquid sample obtained from the treatment reactor at the same growth time before disinfectant was applied. The 6-week and 10-week old samples from the treatment and the control reactors were grouped into two separate clusters. The 6-week and 10-week old samples from the treatment reactor were grouped with the two-week samples and separated from the samples of the same age from the control reactor. The topology of clusters did not change after matrix transformation was applied (Figure 18 B)

The non-metric multi-dimensional scaling (MDS) plotted the biofilm samples on a “map”, where the distance showed the relative similarity of the community structure (Figure 19 A). In this plot, the trend reflected in the cluster analysis in Figure 18 was presented visually. Like cluster analysis, the two-week old samples from the control and the treatment reactors were grouped closely. Biofilm samples obtained after disinfection were plotted in a distinguishably different direction from the samples of same ages without disinfection, suggesting that the
community development processes under these two conditions were different. Applying transformation (Figure 19 B) to the matrix did not affect the topology of MDS result.
5. DISCUSSION AND FUTURE RESEARCH

5.1 Discussion

Persistence of microbial biofilms in post-disinfected drinking water distribution systems is a *de facto* phenomenon. Previous studies on the responses of biofilms to disinfection treatment are typically conducted at a contact time from several minutes to a few hours (De Beer et al., 1994; Huang et al., 1995; Lee et al., 2010). The time scale is comparable to previous reports on the penetration time required for the bulk liquid disinfectants (i.e., free chlorine and monochloramine) to reach the bottom of a biofilm (De Beer et al., 1994; Lu, 2008). However, the development of biofilms in an oligotrophic environment is slow (Martiny et al., 2003) and the penetration of disinfectants would change as exposure time extends. Therefore, monitoring the response of biofilms over a long period is essential to the understanding of biofilm persistence under disinfection, and this question was investigated in this thesis.

In this study, multispecies biofilms were allowed to develop in groundwater, one type of source waters used for the production of drinking water, for up to ten weeks. During this period, monochloramine disinfection with an average concentration of 8.5 mg Cl₂/L as combined chlorine was applied. This exposure time was 5375 times longer than the penetration time determined by Lu (2008) using a similar level of monochloramine concentration applied to biofilms on PVC substratum. Thus, the impact of monochloramine disinfection on biofilm architecture and community development could be investigated.

The findings revealed that long-term disinfection could lead to the development of a thin and compact biofilm structure (Figure 14). In contrast, biofilms developed under the same period without the presence of a disinfectant were observed to contain channels and voids (Figure 12). Under both treatments, biofilm samples taken at different time points were observed to consist of
both live and dead cells. Under a short-term monochloramine treatment, cells in biofilms were observed to lose their respiratory activities first (Stewart et al., 1994; Huang et al., 1995). It is reasoned that cells at the peripheral of the biofilm lose their activities first, before dissociation or detachment of microbial cells takes place, which eventually lead to a reduction in biofilm thickness and biomass when the disinfection is continued.

This study further quantitatively measured the influence of monochloramine on the biofilm architecture. The change in mean thickness as defined by COMSTAT showed a reduction from 1.65±1.57 μm to 0.021±0.014 μm, and a drop in biomass volume from 0.109±0.105 μm³/μm² to 0.022±0.006 μm³/μm². In contrast, both biofilm thickness and biomass volume increased during the development without disinfection. Based on the biovolume and biofilm mean thickness determined in COMSTAT, a new parameter, biofilm compactness (equation i), was derived in order to measure the change in a biofilm during disinfection. With this definition, the compactness of a groundwater-grown biofilm was observed to increase under the disinfection treatment and decrease in the absence of disinfection.

Likewise, it is possible to revisit previous reports on the study of biofilm architecture using COMSTAT. The developments of multispecies biofilms in a model drinking water distribution system (Martiny et al., 2003), in microtiter plates fed with biofilter effluent (Pang and Liu, 2006), and in a monoculture *E. coli* biofilm (Ito et al., 2009) were compared based on compactness. In these examples, different rates in the increment of biomass volume and biofilm average thickness was observed in during biofilm development (Table 4), which means the amount of biomass over a unit volume was not the same. This trend can be well described using biomass compactness, which had a decreasing trend in these cases and reflected the development
of a fluffier biofilm structure. These observations suggest that biofilm compactness can serve as a useful parameter in characterizing biofilm development.

The findings in this study further revealed that the microbial community structure of groundwater biofilms could be influenced by chloramination. Biofilms with a high similarity in community structure before disinfection became dissimilar after disinfection, suggesting that disinfection is an allogenic factor to affect the development of biofilm community. The community structure at the end of this experiment was likely a result from the effect of biofilm growth as well as the cell inactivation and biofilm detachment caused by monochloramine disinfection.

This study revealed that membrane intact cells could present inside the biofilm at the end of a 8-week disinfection, where the biofilm has undergone structural and community change. This phenomenon was compared to findings describing the persistence of live cells after antibiotic treatment in monoculture biofilms (Stewart, 2003; Ito et al., 2009; Harrison et al., 2005). For example, it was observed that live cells could persist in the center of a mushroom-shaped biofilm of *Streptococcus* mutants after treated with a mouthwash (Stewart, 2003), in a flat shaped *E. coli* biofilms after treatments with antibiotics (Ito et al., 2009), and in *P. aeruginosa* biofilm after treatment with metal ions (Harrison et al., 2005). In cases where contact time is much longer than reagent penetration time, persistence can no longer be explained with reduced penetration of antimicrobial agents (Stewart, 2003). It was hypothesized that subpopulations of cells with a “persister” phenotype could develop after the treatment of antimicrobial agents (Lewis 2001). The observations from this study also suggest a possibility that multispecies biofilms developed from groundwater could lead to the development of persisters in addition to microorganisms that are more resistant to monochloramine.
In summary, this study constructed an experimental system to study oligotrophic biofilm development under long-term monochloramine disinfection. The results suggested that high-concentration monochloramine disinfection can control the development of multispecies biofilm growth on PVC surface, shape biofilm architecture, and select a biofilm community that is likely to have resistance to disinfection.

5.2 Future research

This study characterized the impact of monochloramine disinfection on biofilm development in its structure and community. The result indicates that following experiments can be conducted to better understand the impact disinfection on the biological quality of water in distribution systems.

i) Understand the water quality impact of disinfection-triggered detachment of biofilms

Experiments in this study showed a significant decrease in biomass volume and biofilm thickness upon an extended period of disinfection. This indicates that the detachment of biofilms in response to disinfection is an important process associated with disinfection. This phenomenon was previously observed (Codony et al., 2005) but poorly understood. To better understand the detachment process triggered by disinfection, the microbial community, viability and biofilm forming potential of the detached cells need to be further characterized. This understanding can be used to predict the impact of disinfection, for example whether detached biofilms would become seedings for new biofilms in distribution systems.

ii) Characterize residual biofilms after monochloramine disinfection

The biofilms survived under an extended period of monochloramine disinfection presented in this study calls for a better understanding of the resistance of multispecies biofilms
against disinfection. With the community structure difference presented with T-RFLP, the microbial community of biofilm persisting disinfection can be further characterized with pyrosequencing techniques to better understand the community component and structure. It is also important to further study the gene expression profile of the persistent biofilms, and identify the highly expressed genes.
6. FIGURES AND TABLES

Figure 1. Developmental model of biofilm formation (modified from Monds and O’toole 2009)

Figure 2. Experiment Schematics

Community analysis
Terminal restrictive fragment length polymorphism (T-RFLP)
DNA extraction:
Schmidt protocol
PCR
Amplify 16S rRNA gene with 47F-FAM and 927R
Enzymatic digestion
HhaI, MspI
Electrophoresis
ABI 3730 genetic analyzer
Peak binning and data processing
T-RFLPstat

Biofilm architecture
Stain the biofilm with fluorescent dyes
SYTO-9: all cells
PI: dead cells
Observe under confocal laser scanning microscope
LSM 710
Image analysis
Obtain biovolume and thickness with COMSTAT
Figure 3. Reactor configuration. (A) Reactor and reservoir, (B) CDC reactor, (C) Coupons and holding rod.
Figure 4. Comparison of *MspI* and *HhaI* on digestion of biofilm samples before and after chloramine disinfection.
Figure 5. Disinfection operation in the 4-week experiment. The plot presents influent disinfectant concentration in the treatment reactor. Arrows indicate days when biofilm samples were collected. Samples were taken from the control reactor at the same time points.
Figure 6. T-RFLP fingerprints of biofilm samples from control reactor (A, C, E and G) and treatment reactor (B, D, F, and H). Samples were collected at reactor operation for 2 weeks (A and B), 16 days (C and D), 3 weeks (E and F), and 4 weeks (G and H). Peaks common to each reactor was marked with fragment length on the graphs.
Figure 7. Cluster analysis based on T-RFLP data sets from the 4-week experiment. Similarity between samples was calculated without conducting matrix transformation (A) or after presence/absence transformation (B). C: control reactor; T: treatment reactor
A. Without transformation

**4 week experiment**

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-16 day</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>T-3 week</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>T-4 week</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>C-2 week</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>C-3 week</td>
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<tr>
<td>C-4 week</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>T-16 day</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>T-3 week</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>C-16 day</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>T-2 week</td>
<td>▲</td>
<td>○</td>
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B. Presence/absence transformation

**4 week experiment**

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<thead>
<tr>
<th>Reactor</th>
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<tr>
<td>T-4 week</td>
<td>▲</td>
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<td>C-2 week</td>
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<td>○</td>
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<tr>
<td>C-3 week</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>C-16 day</td>
<td>▲</td>
<td>○</td>
</tr>
<tr>
<td>T-16 day</td>
<td>▲</td>
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</tr>
<tr>
<td>T-3 week</td>
<td>▲</td>
<td>○</td>
</tr>
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</table>

Figure 8. Non-metric multidimensional analysis of T-RFLP data from the 4-week experiment. Similarity between samples was calculated without conducting matrix transformation (A) or after presence/absence transformation (B). C: control reactor; T: treatment reactor.
Figure 9. Disinfection operation in the 10-week experiment. Both influent (■-influent total chlorine, ▲-influent free chlorine) and reactor disinfectant concentrations (□-reactor total chlorine, △-reactor free chlorine) were presented. Arrows indicate time points that biofilm samples were collected. Samples were taken from the control reactor at the same time points. Bulk liquid samples from the reactor were taken at the second week and tenth week.
Figure 10. pH (A) and DO (B) in the bulk liquid of the control and treatment reactor.
Figure 11. CLSM images showing biofilm architectural development in control reactor. Samples were taken at reactor operation for 2 weeks (A and B), 6 weeks (C and D), and 10 weeks (E and F). Images were presented as extended focus images (A, C and E) and 3-D images (B, D, and F). Green signals are SYTO-9 stained cells which present total cells, while red signals are PI stained cells which present membrane damaged cells. Arrows on the images indicate the presence of aggregates of cells.
Figure 12. Reflected-light confocal laser scanning image for mature biofilms without disinfection. Blue color represents signals obtained under reflective mode.
Figure 13. CLSM images showing biofilm architectural development in treatment reactor. Samples were taken at reactor operation for 2 weeks (A and B), 6 weeks (C and D), and 10 weeks (E and F). Images were presented as extended focus images (A, C and E) and 3-D images (B, D, and F). Colors of signals are the same as in Figure 11. Images from disinfected biofilms (6-week and 10-week) were weak in fluorescent signals under microscope. 10-week biofilms were taken under laser compensation to generate optimal images.
Figure 14. Reflected-light confocal laser scanning image for biofilms 8 weeks after disinfection. Blue color presents signals obtained under reflective mode.
Figure 15. Quantitative analysis of biofilm architectural development. Panels A, C, and E present biomass volume, average thickness, and maximum thickness development in the control reactor. Panels B, D, and F present the temporal changes of the same parameters in the treatment reactor.
Figure 16. Temporal change in compactness as biofilms developed in control (A) and treatment reactors (B).
Figure 17. T-RFLP fingerprints from the 10-week experiment. Samples were collected from control (A, C, and E) and treatment reactor (B, D, and F) after 2 weeks (A and B), 6 weeks (C and D), and 10 weeks (E and F) of operation. Peaks marked in the figure are dominant peaks present in one or more samples.
Figure 18. Cluster analysis for T-RFLP fingerprints from the 10-week experiment. Similarity between samples was calculated without conducting matrix transformation (A) or after presence/absence transformation (B). C: control reactor; T: treatment reactor C: control reactor; T: treatment reactor
Figure 19. Non-metric multidimensional scaling result from the 10-week experiment. Similarity between samples was calculated without conducting matrix transformation (A) or after presence/absence transformation (B). C: control reactor; T: treatment reactor
Table 1. Primer information

<table>
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<th>Specificity</th>
<th>Position</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
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<td>47F(6-FAM)</td>
<td><em>Bacteria</em></td>
<td>16S, 47-66</td>
<td>CYT AAC ACA TGC AAG TCG</td>
<td>Chen et al., 2004</td>
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<tr>
<td>927R</td>
<td><em>Bacteria</em></td>
<td>16S, 927-942</td>
<td>ACC GCT TGT GCG GGC CC</td>
<td>Giovannoni, et al., 1988</td>
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</tbody>
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Table 2. Groundwater quality

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<td>Ca (total)</td>
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<tr>
<td>Fe (total)</td>
<td>0.623 (0.363)</td>
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<tr>
<td>Mg (total)</td>
<td>24.8 (1.5)</td>
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<tr>
<td>Mn (total)</td>
<td>3.33 (1.48)</td>
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<tr>
<td>orthophosphate-P</td>
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<tr>
<td>NVOC</td>
<td>4.03 (0.77)</td>
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<td>NH₃-N</td>
<td>1.09 (0.19)</td>
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<td>NO₂⁻-N</td>
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<td>NO₃⁻-N</td>
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Table 3. Live and dead stain results in two reactors.

<table>
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<tr>
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<th>Green/red ratios (standard deviation)</th>
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<td>Reactor</td>
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</tr>
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</tr>
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<td>Control</td>
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<td>Control</td>
<td>10 week</td>
</tr>
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<td>Treatment</td>
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<td>Treatment (compensated)</td>
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<tr>
<td>Treatment (non-compensated)</td>
<td>10 week</td>
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<tr>
<td></td>
<td>Biomass volume (μm³/μm²)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td><strong>Multispecies biofilm developed in a pilot drinking water distribution system (Martiny et al., 2003)</strong></td>
<td></td>
</tr>
<tr>
<td>Young biofilm (1-94 d)</td>
<td>1.6</td>
</tr>
<tr>
<td>Old biofilm (500-1093 d)</td>
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<td><strong>E.coli biofilm developed in a flow cell fed with MOPS minimal medium with glucose (Ito et al., 2009)</strong></td>
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<td>maturation (72h)</td>
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<td>mature biofilm after air flushing</td>
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REFERENCES


United States Environmental Protection Agency, 1998. Small system compliance technology list for the surface water treatment rule and total coliform rule.