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THE ROLE OF BIOFILMS IN
LEGIONELLA PNEUMOPHILA TRANSMISSION IN DRINKING
WATER DISTRIBUTION SYSTEM

BY

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DISSERTATION

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Biofilms in drinking water distribution systems (DWDS) or premise plumbing systems could facilitate the persistence and transmission of pathogenic *Legionella pneumophila* (*L. pneumophila*), thus raise human health concerns. *L. pneumophila* cells can accumulate in biofilms, be protected from disinfection by biofilms, and then be released from biofilms with detached biofilm materials or by the drinking water flow shear stress. Biofilm properties (e.g., physical structure and mechanical stiffness) play an important role during this *L. pneumophila* transmission process. However, the knowledge on how the biofilm properties control *L. pneumophila* transmission and what factors in DWDS determine biofilm properties is still unclear. Therefore, this research aimed to 1) identify the key factors controlling biofilm-associated *L. pneumophila* accumulation, persistence, and release; 2) investigate how the biofilm structural, mechanical, and chemical properties vary in response to a complex DWDS environment.

First, this research identified the effect of biofilm structure on *L. pneumophila* adhesion to and release from simulated drinking water biofilms. Roughness of biofilms was found to enhance the adhesion of *L. pneumophila* to biofilms due to enlarged biofilm surface area and local flow conditions created by roughness asperities. However, the release of *L. pneumophila* from biofilms was prevented by biofilms, presumably because of low shear stress zones near roughness asperities. Next, the effect of disinfectant exposure, an important parameter of drinking water quality, on biofilm structure and stiffness as well as the corresponding pathogen release and inactivation was identified. The biofilm thickness recovered during a long-term disinfectant exposure, indicating that the long-term disinfection could not significantly remove biofilms. However, the biofilms became stiffer after long-term disinfection. By using the simulated drinking water containing
disinfectant to release the adhered *L. pneumophila* from the stiffened disinfected biofilms, the inactivation and infectivity of released *L. pneumophila* was examined. Compared to non-disinfected (softer) biofilms, the *L. pneumophila* released from disinfected (stiffer) biofilms showed higher inactivation ratio and lower infectivity. Therefore, those stiffened biofilms provided less protection for the biofilm-associated *L. pneumophila* under disinfectant exposure. Lastly, the role of drinking water scaling control (e.g., hardness reduction and scale inhibitor application) on the chemical composition, physical structure, and mechanical stiffness of biofilms was investigated. Applying the scale control to water source diminished the calcium carbonate precipitating inside biofilms, thus led to biofilms with low stiffness. Notably, application of scale inhibitor (polyphosphate) produced the thickest biofilms. High pathogen release would be expected from those thick and soft biofilms developed in present of polyphosphate.

This research comprehensively investigated the accumulation, disinfection, and release of *L. pneumophila* associated with biofilms under the continuous drinking water flow and disinfectant exposure conditions, which best mimicked the DWDS in practice. The results of this study highlighted the relation between biofilms, pathogens, and drinking water, thus could provide information on risk assessment of control of pathogens in DWDS.
To My Father and Mother,

Who Taught Me How to Love This World
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CHAPTER 1: INTRODUCTION

1.1 Background

*Legionella pneumophila* (*L. pneumophila*), the main causative agent of legionellosis, was commonly found in both natural fresh water environment and human-made water systems. The water containing *L. pneumophila* can be aerosolized and inhaled by human body, thus cause *L. pneumophila* infection. During 2011-2013, 1426 legionellosis cases were reported and 9% of patients with Legionellosis died according to the report from Centers for Disease Control and Prevention (CDC). Drinking water is an important medium to transmit *L. pneumophila*. From 2011 to 2012, 21 disease outbreaks caused by *L. pneumophila* in drinking water were reported, contributed to 66% of the total reported disease outbreaks associated with drinking water in United States. Therefore, controlling the transmission of *L. pneumophila* in drinking water is a key to preventing the disease outbreaks of legionellosis.

*L. pneumophila* can transmit and persist in drinking water distribution system (DWDS) with the aid of biofilms. Biofilms, composed by microorganisms, extracellular polymeric substances (EPS), and inorganic particles, are ubiquitous in DWDS and premise plumbing systems. Once *L. pneumophila* cells intrude into the drinking water systems through pipe damage, cross-connections or operation disturbances, biofilms can capture these *L. pneumophila* cells and provide a favorable environment for *L. pneumophila*. *L. pneumophila* can be resistant to extreme pH and temperature, oligotrophic, and disinfectant environment when co-exists with biofilm. Biofilm provides nutrients and energy sources to support the growth and propagation of *L. pneumophila*. Although disinfectant residual is required in most drinking waters in US, biofilms were reported to protect *L. pneumophila* from disinfection.
Subsequently, these biofilm-associated *L. pneumophila* cells can be released with the sloughed off biofilm or by the drinking water flow. The released *L. pneumophila* cells would recolonize downstream biofilms or reach consumers, which may increase risk for *L. pneumophila* exposure and infection.\(^{22}\) However, although biofilms take an important role on *L. pneumophila* transmission, how biofilm properties affect *L. pneumophila* accumulation, inactivation, and release in the complex drinking water environment is still unclear. Therefore, comprehensive understanding of the interaction among biofilms, drinking water, and *L. pneumophila* transmission is necessary for *L. pneumophila* control in DWDS.

The accumulation and release of *L. pneumophila* associated with biofilms in DWDS could be influenced by biofilm physical structure and mechanical properties. For example, the roughness of biofilms was found to enhance the adhesion of E.coli on mono-species or multi-species biofilms.\(^{23,24}\) However, the knowledge on how does biofilm roughness work on enhancing bacteria adhesion and if the roughness could also influence bacteria release is still needed. In addition, the *L. pneumophila* and other pathogens can release from the biofilms during the biofilm detachment process. The biofilm mechanical properties (e.g., elasticity and cohesiveness) have been shown to be essential factors controlling the detachment of biofilms,\(^{25-27}\) thus may affect the release of biofilm-associated pathogens. Nevertheless, the knowledge on relationship between biofilm mechanical properties and pathogen release is still lacking.

The inactivation of *L. pneumophila* and other pathogens associated with biofilms under drinking water disinfectant exposure can also be influenced by biofilms. The composition of biofilms were reported to affect the disinfection efficacy of biofilm-associated *L. pneumophila* or other pathogens.\(^{28-32}\) For example, the biofilms composed by mono-species *Microbacterium phyllosphaerae* or *L. pneumophila* were shown to be less tolerant to hydrogen peroxide or
dendrimers exposure than the mixed-species biofilms.\textsuperscript{31, 32} While biofilms can protect pathogens by blocking the transfer of disinfectant to reach \textit{L. pneumophila},\textsuperscript{12, 33} the diverse biopolymers produced by multi-species biofilms may lead to a more viscous biofilm matrix and thus reduce the diffusion of disinfectant in biofilms.\textsuperscript{32, 34} However, most of these previous studies investigated the disinfection for biofilm-associated pathogens under quiescent conditions. While the drinking flow shear stress may improve the disinfectant transfer in biofilms and detach the pathogens from biofilms,\textsuperscript{35, 36} the role of biofilm in pathogen disinfection under flow condition and the concurrent pathogen release has not been studied. In addition, previous studies showed that planktonic \textit{L. pneumophila} can still maintain the ability to infect their host, \textit{Acanthamoeba castellanii}, 4 month after monochloramine treatment.\textsuperscript{37} However, the role of biofilms in the infectivity of biofilm-associated pathogen after disinfection treatment was not investigated before.

While the biofilm properties affect \textit{L. pneumophila} transmission in DWDS, the knowledge on how the factors from complex drinking water environment influence the biofilm properties is lacking. Specifically, the disinfectant residuals may influence the biofilm mechanical and structural properties through biomass loss and change in biofilm chemical composition. Previous studies showed the reduced biofilm thickness and stable biofilm cohesiveness during short-term (e.g., days or hours) disinfectant exposure.\textsuperscript{38, 39} However, it is unknown how long-term disinfectant exposure may influence the chemical composition, structure, and mechanical properties. In addition, scale control strategies (e.g., water hardness reduction and scale inhibitor application) are applied in drinking water to prevent the pipe blocking caused by scales (e.g., calcite). These scales on pipe walls co-existed with biofilms\textsuperscript{40} are possible to affect the composition, physical structure, and mechanical properties of biofilms. In addition, some scale inhibitors, such as polyphosphate, can support the growth of bacteria thus facilitate the biofilm accumulation,\textsuperscript{16} thus
influence the biofilm composition and structure. However, the role of water hardness and scale inhibitors on biofilm properties is still unclear. Therefore, how the water components (disinfectant, hardness, and scale inhibitors) affect biofilm chemical composition, physical structure, and mechanical properties need to be investigated.

1.2 Research Objectives

The overall goal of this research is to elucidate the role of biofilms in the accumulation (adhesion), disinfection, and release of L. pneumophila in DWDS. Specifically, this research aimed to investigate how biofilm properties varied in response to a complex drinking water environment as well as their contribution to L. pneumophila transmission. The results of this research can provide insights to predict, assess, and aid in controlling the risk of pathogens associated with DWDS biofilms. The specific objectives of this research are:

1. To determine the role of biofilm roughness and the local hydrodynamics created by roughness in L. pneumophila adhesion to and release from biofilms. To mimic the pathogen adhesion and release in real DWDS, the L. pneumophila adhesion to biofilms under low flow velocity and L. pneumophila release from biofilms under high flow velocity was determined. The biofilms with different roughness was used in Legionella adhesion and release experiments.

2. To investigate the effect of long-term disinfectant exposure on biofilm physical structure and stiffness. To explore how the biofilm response to disinfectant exposure, the biofilm physical structure and stiffness during three months of monochloramine or free chlorine exposure was monitored.
3. To evaluate the release, inactivation, and infectivity of biofilm-associated *L. pneumophila* under disinfectant exposure. To evaluate the role of biofilms in *L. pneumophila* disinfection under the flowing drinking water condition, inactivation and infectivity of *L. pneumophila* released from biofilms were quantified. Both of the pre-disinfected and non-disinfected biofilms from Objective 2 were used here, and their contribution on protecting *L. pneumophila* from disinfection was compared.

4. To identify the effect of water hardness and scale inhibitor on biofilm chemical composition, physical structure, and stiffness. The composition, structure, and stiffness of biofilms developed from high hardness groundwater, softened groundwater, and groundwater containing scale inhibitor was determined.

### 1.3 Dissertation Organization

In Chapter 2, entitled “role of biofilm roughness and hydrodynamic conditions in *Legionella pneumophila* adhesion to and detachment from simulated drinking water biofilms”, we 1) experimentally quantified *L. pneumophila* adhesion on biofilms with different roughness and used computational fluid dynamics (CFD) to reveal the role of hydrodynamics created by surface roughness; 2) identified the effect of biofilm roughness and hydrodynamics on detachment (release) of pre-adhered *L. pneumophila*.

The results of Chapter 2 indicated that biofilm roughness can enhance the adhesion of *L. pneumophila* on biofilms due to enlarged biofilm surface area and local flow conditions created by roughness asperities. Also, the biofilm roughness can prevent *L. pneumophila* releasing from
biofilms, presumably because of the low shear stress zones near roughness asperities. These results identified the importance role of biofilm structure in \textit{L. pneumophila} transmission. Next, the biofilm structure and other properties relevant to pathogen transmission were further studied in the following chapters.

In Chapter 3, entitled “response of simulated drinking water biofilm mechanical and structural properties to long-term disinfectant exposure”, we monitored the structure and mechanical properties of simulated drinking water biofilms during three months of disinfectant exposures. We measured biofilm stiffness with atomic force microscopy (AFM) and biofilm structure (thickness and roughness) with OCT to determine the role of disinfectant exposure, shear conditions, and exposure duration time on biofilm mechanical and structural properties.

The results of Chapter 3 indicated that biofilms were stiffened and thinned within the relative short term (one month) of disinfectant exposure. However, the biofilms became softer and thicker again with longer disinfectant exposure, suggesting that biofilms can adapt to disinfectant exposure. Overall, long-term disinfectant exposure did not reduce biofilm thickness, but increased the biofilm stiffness. The corresponding \textit{L. pneumophila} transmission associated with those disinfected biofilms was further discussed in Chapter 4.

In Chapter 4, entitled “effect of disinfectant exposure on Legionella pneumophila associated with simulated drinking water biofilms: release, inactivation, and infectivity”, we examined the inactivation and infectivity of \textit{L. pneumophila} released from biofilms under a continuous disinfectant exposure and flow condition. Both long-term pre-disinfected and non-disinfected biofilms from Chapter 3 were used here. The effect of pre-disinfecting biofilms and disinfectant species on the disinfection and release of biofilm-associated \textit{L. pneumophila} was identified.
The results of Chapter 4 showed that under disinfectant exposure, the inactivation of \textit{L. pneumophila} released from pre-disinfected biofilms was higher than that from non-disinfected biofilms; while the infectivity of \textit{L. pneumophila} released from pre-disinfected biofilms was lower than non-disinfected biofilms. Non-disinfected biofilms can provide better protection for \textit{L. pneumophila} under disinfectant exposure, probably because more biofilm materials detached from the softer non-disinfected biofilms, surrounded the released \textit{L. pneumophila}, and separated the \textit{L. pneumophila} from disinfectant.

In Chapter 5, entitled “effect of water hardness and scale inhibitors on chemical composition, physical structure, and mechanical stiffness of simulated drinking water biofilms”, the composition, structure, and stiffness of biofilms developed from high hardness groundwater, softened groundwater, and groundwater containing scale inhibitor was determined.

The results of Chapter 5 indicated that the biofilms developed from groundwater had the highest content of calcium carbonate, thus showed the highest stiffness among all the examined biofilms. While the scale inhibitor prevented the crystallization of calcium carbonate and facilitate the growth of microorganisms, soft and thick biofilms were developed from groundwater containing scale inhibitor.

1.4 References


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CHAPTER 2: ROLE OF BIOFILM ROUGHNESS AND HYDRODYNAMIC CONDITIONS IN LEGIONELLA PNEUMOPHILA ADHESION TO AND DETACHMENT FROM SIMULATED DRINKING WATER BIOFILMS

2.1 Abstract*

Biofilms in drinking water distribution systems (DWDS) could exacerbate the persistence and associated risks of pathogenic Legionella pneumophila (L. pneumophila), thus raising human health concerns. However, mechanisms controlling adhesion and subsequent detachment of L. pneumophila associated with biofilms remain unclear. We determined the connection between L. pneumophila adhesion and subsequent detachment with biofilm physical structure characterization using optical coherence tomography (OCT) imaging technique. Analysis of the OCT images of multi-species biofilms grown under low nutrient condition up to 34 weeks revealed the lack of biofilm deformation even when these biofilms were exposed to flow velocity of 0.7 m/s, typical flow for DWDS. L. pneumophila adhesion on these biofilm under low flow velocity (0.007 m/s) positively correlated with biofilm roughness due to enlarged biofilm surface area and local flow conditions created by roughness asperities. The pre-adhered L. pneumophila on selected rough and smooth biofilms were found to detach when these biofilms were subjected to higher flow velocity. At the flow velocity of 0.1 and 0.3 m/s, the ratio of detached cell from the smooth biofilm surface was from 1.3 to 1.4 times higher than that from the rough biofilm surface, presumably because of the low shear stress zones near roughness asperities. This study determined that physical structure

and local hydrodynamics control adhesion and detachment from simulated drinking water biofilm, thus it is the first step toward reducing the risk of L. pneumophila exposure and subsequent infections.

2.2 Introduction

Biofilms are ubiquitous in drinking water distribution systems (DWDS). The presence of biofilm potentially increases the persistence and associated risks of pathogens.\(^1\text{-}^4\) DWDS biofilms provide a favorable environment for capture, growth, propagation, and release of pathogens, such as Legionella pneumophila (L. pneumophila), by supplying nutrients\(^5\text{-}^9\) and protecting pathogens from disinfection.\(^10\text{-}^12\) L. pneumophila is known as the main causative agent of legionellosis,\(^13\) which is reported worldwide. In the United States, 3688 legionellosis disease cases were reported in 2012.\(^14\) L. pneumophila contributed to 58% of total waterborne disease outbreaks associated with US drinking water between 2009 and 2010.\(^15\) In Europe, 5952 legionellosis disease cases were reported by 29 countries in 2012. The investigation conducted for some of these cases found that water distribution system contributed to 62% of all sampling sites with positive L. pneumophila test results.\(^16\) While DWDS biofilms can harbor L. pneumophila, the role of biofilms in accumulation and release of L. pneumophila is still largely overlooked. Notably, adhesion (capture) of L. pneumophila to biofilms is a prerequisite of L. pneumophila persistence and propagation, and subsequent detachment (release) of L. pneumophila from biofilms under high flow results in the increased risks of L. pneumophila exposure and infection.\(^17\) Therefore, comprehensive understanding of L. pneumophila adhesion and detachment associated with
biofilms will elucidate the factors affecting *L. pneumophila* transmission to humans and provide guidelines for *L. pneumophila* risk control in DWDS.

Chemical (e.g., solution ionic strength) and physical (e.g., biofilm roughness and flow conditions in DWDS) factors may control adhesion and detachment of *L. pneumophila* and other pathogens associated with biofilms. Increasing ionic strength was believed to control bacteria adhesion on a variety of surfaces (Teflon, glass, protein coated glass, and other surfaces) through reducing the electrostatic repulsion between bacteria and the surface.\textsuperscript{18-21} However, on single or multi-species biofilms, ionic strength was found to have little to no effect on adhesion of *E. coli* and *Erwinia chrysanthemi*,\textsuperscript{22, 23} indicating that electrostatic interactions did not control adhesion on biofilms. Thus, the effects of physical factors on bacteria adhesion on biofilms should be studied, but were addressed in only limited studies. For example, unevenness of a surface, which is referred to as surface roughness, was found to influence *E. coli* adhesion on *Pseudomonas aeruginosa* biofilms\textsuperscript{24} and multi-species biofilms.\textsuperscript{23} However, mechanisms of how biofilm roughness affects *L. pneumophila* and other bacteria adhesion and if biofilm roughness affects bacteria detachment were unknown. In addition to biofilm roughness, hydrodynamic conditions were also shown to influence cell adhesion to and detachment from multiple surfaces.\textsuperscript{25-28} High shear stress caused by high flow velocity prevented cell adhesion onto the clean and smooth surfaces,\textsuperscript{25, 27} and enhanced detachment of biofilms.\textsuperscript{25, 28, 29} Nevertheless, for heterogeneous rough biofilm surfaces, local hydrodynamics could be disturbed by the surface asperities. This local hydrodynamics created by surface asperities may alter the adhesion and detachment of *L. pneumophila* and other bacteria associated with biofilms and should be investigated. However, previous studies on *L. pneumophila* adhesion and detachment did not address the effect of biofilm physical properties nor hydrodynamics conditions.\textsuperscript{30, 31} Therefore, a comprehensive study identifying the combined effect
of surface roughness and hydrodynamics on \textit{L. pneumophila} adhesion and detachment is needed to understand \textit{L. pneumophila} transmission in DWDS.

To fill the aforementioned research gaps, we determined the physical structure of groundwater biofilms under different flow conditions and the influence of these structures on the mechanisms of \textit{L. pneumophila} adhesion and detachment. Specifically, we 1) used optical coherence tomography (OCT) to determine whether the biofilm deform when being exposed to flow with velocity up to 0.7 m/s; 2) experimentally quantified \textit{L. pneumophila} adhesion on biofilms under low flow condition and used computational fluid dynamics (CFD) to reveal the role of hydrodynamics created by surface roughness; 3) identified the effect of biofilm roughness and hydrodynamics on detachment of pre-adhered \textit{L. pneumophila}. This study sheds light on the mechanism affecting \textit{L. pneumophila} adhesion to and detachment from biofilms, which are likely key steps in the transmission of the legionellosis disease from DWDS.

2.3 Materials and Methods

2.3.1 Biofilm preparation

A local groundwater source, which is also a source for drinking water in Urbana-Champaign, IL, was selected for growing biofilms in this study. The microbial communities from the groundwater and the time required for biofilm development have been previously characterized.\textsuperscript{23, 32} PVC coupons (RD 128-PVC, BioSurface Technologies Corporation, Bozeman, MT) with the diameter of 1.26 cm were selected as the substratum of biofilm because PVC is a common plastic material used for drinking water pipes. Biofilms were grown on PVC coupons in CDC reactors (CBR 90-
2, BioSurface Technologies Corporation, Bozeman, MT) with continuous stirring at 125 RPM or Re of 2384 as described previously.23

2.3.2 L. pneumophila cell preparation

*L. pneumophila* (ATCC® 33152™) tagged with green fluorescence protein (GFP) by electroporating plasmids pBG307 was used in this study.33 *L. pneumophila* cells were grown in buffered yeast extract medium, harvested and re-suspended in potassium chloride (KCl) solutions for subsequent adhesion experiments. More details of *L. pneumophila* culturing and characterizing are documented in the appendix.

2.3.3 Adhesion experiment and Sherwood number calculation

Adhesion experiments of GPF-tagged *L. pneumophila* cells on unstained 2, 4, 8, 14 and 29-week biofilms and PVC surfaces were conducted using a parallel plate flow chamber (FC 71, BioSurface Technologies Corporation, MT). During each experiment, electrolyte solution with 1-5×10⁷ cells/mL of *L. pneumophila* was pumped into the flow chamber at an average flow velocity of 0.007 m/s with Re of 1.26 for 30 minutes. This average flow velocity was kept constant for all experiments to simulate near stagnant laminar flow conditions in a DWDS, when the highest adhesion of planktonic bacteria to solid surface is expected.25, 34 Measurements over DWDS in Ohio and Arizona found up to 35% and 16% of the total pipe carrying water in laminar flow region, respectively.35, 36 Ionic strengths ranging from 3 to 300 mM were selected to determine the role of electrostatic interactions on adhesion. The number of *L. pneumophila* cells adhering to biofilms
was determined with the aid of a fluorescence microscope or a confocal laser-scanning microscope (CLSM). For experiments using the fluorescence microscope (Leica DM15000 M), the images of the biofilm surface with adhered cells were taken at 1 min intervals throughout the 30 minutes’ adhesion experiments, and the number of adhered cells was counted from each image. For each combination of biofilm age and ionic strength, adhesion experiments were conducted with three biofilms. The imaging area of 0.395×0.296 mm in the center of each biofilm coupon was chosen. For experiments with CLSM (TCS SP2 RBB, Leica Microsystems), real-time determination of adhered cells was not possible because this method requires time to scan the biofilm at different depths. Instead, the 3-dimensional image of adhered cells through the whole biofilm body was obtained. The number of total adhered cells after 30 minutes’ of adhesion process was determined by the 3-D image.

The adhesion was expressed as Sherwood numbers, which represent the average local particle transfer rate to the collector surface. The Sherwood number was calculated as the ratio of experimentally determined cell adhesion mass transfer divided by diffusive mass transfer of the cells, and used to present adhesion data so that the dataset obtained could be compared with previous work. More details of the flow chamber dimension, adhesion experiments and the Sherwood number calculation were described in the appendix.

2.3.4 Detachment experiment

The detachment of pre-adhered *L. pneumophila* from a relatively smooth biofilm and rough biofilm with the relative roughness coefficient of 0.17 and 0.27, respectively, was determined for the average flow velocities of 0.1, 0.3, and 0.7 m/s. These flow velocities correspond to Re of 1.26,
50, and 126. The highest flow velocity was selected to match the design flow rate of 11.4 L/min (3 GPM) of some states in the United States and a common shower pipe size of 0.75 inch or 1.9 cm.\textsuperscript{42, 43} \textit{L. pneumophila} cells were allowed to attach onto the biofilm surface for 30 min at 0.007 m/s, as described in the adhesion experiment. A 3 mM KCl solution free of \textit{L. pneumophila} was then introduced into the flow chamber at 0.007 m/s to wash the flow chamber and remove \textit{L. pneumophila} cells floating above the biofilm surface. After washing the flow chamber for 20 min, the average flow velocity was increased to promote the detachment of adhered \textit{L. pneumophila} cells from biofilms. The detachment process under each flow condition during a period of 30 min was recorded using a fluorescence microscope at intervals of 1 min. The number of retained cells on biofilm surfaces at each imaging time point was counted. The ratio of retained cells ($R_t$), final detached cell ratio ($D_{\text{final}}$), and the time for 90% of maximal cell detachment ($T_{90}$) was determined and described in the appendix.

2.3.5 OCT image collection and structure analysis for biofilms

Optical coherence tomography (OCT) was used to determine the roughness and thickness of the different biofilms. For OCT measurements, the coupons were removed from the CDC reactors and placed in a flow chamber, which was also used for adhesion and detachment experiments. Biofilm images were captured by a spectral-domain OCT system, which utilized a mode-locked titanium:sapphire laser source (Kapteyn-Murnane Laboratories, Inc, Boulder, CO) centered at 800 nm with a 120 nm bandwidth. Axial and transverse imaging resolution was 1.8 $\mu$m and 16 $\mu$m. Two-dimensional cross-sectional images were acquired at a 25 Hz imaging rate with 1000 A-scans (columns) per image. Biofilm mean thickness, relative roughness coefficient, and biofilm surface
enlargement coefficient\textsuperscript{44} was obtained by analyzing 20-25 OCT images for a given biofilm with the program developed by Derlon et al\textsuperscript{45} and described in the appendix.

As a control experiment to identify the possible biofilm structure deformation under the flow conditions used in the adhesion experiments, OCT images were taken for a selected mature biofilm when continuously exposed to different average flow velocities (0, 0.007, and 0.03 m/s) in the flow cell. For monitoring the possible biofilm structure change under high flow rate used in detachment experiments, both the 30- (rough) and 34-week (smooth) biofilms were continuously imaged by OCT for half an hour when the 3 mM KCl solution was introduced to the flow cell at the flow velocities of 0.1, 0.3, and 0.7 m/s. Each measurement was repeated three times on different biofilm coupons from the same reactor.

\textit{2.3.6 CFD and particle tracing simulation for flow across the biofilms}

Ten rough (4-week, relative roughness coefficient = 0.76±0.07) and ten smooth (14-week, relative roughness coefficient = 0.30±0.07) biofilm 2-dimensional contours obtained from OCT imaging were used for the simulation of velocity distribution and particle movement above the biofilm surface in the flow chamber. The simulation was conducted with COMSOL Multiphysics 4.3a (Comsol Inc, Burlington, MA) and had two steps. For the first step, the Navier-Stokes equation for flow profiles inside the flow chamber was numerically solved with a no-slip boundary condition on both biofilm surfaces and the glass cover slide wall. The initial velocity was set as the average flow velocity (0.007 m/s) inside the flow cell. In the second simulation step, spherical particle movement in this flow field was simulated based on Newtonian’s law of motion, drag
force, and Brownian motion. Drag force was calculated from Stokes equation and flow velocity. Brownian motion was determined by particle size (2 µm), dynamic viscosity, and a random number generator factor for particle diffusion. 1000 particles were continuously delivered together with the fluid into the flow chamber for 10 seconds. These particles were dispersed in the flow by the drag force and Brownian motion. Finally, the adhesion of particles was represented by deposition probability, which was calculated by dividing the final number of adhered particles with the number of total released particles. The simulation was conducted in the fluid phase, and the flow was at steady state.

The Navier-Stokes equation was also solved numerically with no-slip boundary conditions for all average flow velocities (0.1, 0.3, and 0.7 m/s) used in detachment experiments for the selected rough and smooth biofilm OCT contours. Shear stress distribution, a critical factor controlling the detachment of *L. pneumophila* from biofilm, was calculated based on these velocity profiles. This shear stress simulation was time-independent. More physical parameters used in particle tracing and shear stress simulation are in the appendix (Table A.5).

2.3.7 *Statistical analysis*

Statistical analysis was conducted for all Sherwood numbers obtained from fluorescence microscope and CLSM adhesion experiments. The significance level of 0.05 was used for both one way ANOVA and t-test. See the appendix for more details.
2.4 Results and Discussion

2.4.1 Biofilm structure determined by OCT imaging

The effects of biofilm age on its thickness and roughness were determined under no flow conditions. The average biofilm thickness increased with age, from 20±4 µm for a 4-week biofilm to 38±5 µm for a 14-week biofilm. After 14 weeks, the biofilm thickness stabilized. Specifically, the average thickness between a 29-week biofilm (32±14 µm) and a 14-week biofilm (38±5 µm) was similar (α=0.05, p=0.22). The highest relative roughness coefficient of 0.76±0.07 was observed for the 4-week biofilm. The relative roughness coefficient decreased with the biofilm age to 0.30±0.07 at 14-week. At the 29th week, the roughness increased to 0.67±0.13. These biofilm thickness and roughness values are listed in Table A.1. Overall, the change of biofilm roughness was not correlated with its thickness.

Possible biofilm deformation due to flow through the experimental chamber containing the biofilms was investigated under two flow regimes using OCT imaging. For the low flow conditions, when the flow velocity increased from 0 to 0.03 m/s, the biofilm contours at the same location did not show deformation (Figure 2.1). The average biofilm thickness and roughness at different locations under different flow velocities were statistically similar (Table A.2). Therefore, the effect of biofilm structural change during the adhesion experiments and particle tracing simulation, which used a flow velocity of 0.007 m/s, was not considered.
Figure 2.1 OCT image of 8-week biofilm sequentially exposed to the average flow velocity of 0, 0.007, and 0.03 m/s. The yellow line is drawn manually and shows the boundary between the biofilm and water. These images were taken at the same location on biofilms when the biofilms were subjected to the flow with increasing velocity from 0 to 0.03 m/s.

For the high flow conditions, a relatively rough biofilm and a smooth biofilm with roughness coefficients of 0.27 and 0.17, respectively, were imaged by OCT during continuous exposure to the average flow velocities of 0.1, 0.3, and 0.7 m/s for half an hour. At all flow conditions used here and in the detachment experiment, OCT (with vertical resolution of 2.8 \( \mu \text{m} \) under flow condition) did not detect significant structural deformation for both 30- and 34-week biofilms. For example, the 30-week biofilm contours at the beginning and the end of detachment experiments under different average flow velocities are shown in Figure 2.2. After 30 minutes of exposure time to flow velocities of 0.1, 0.3, and 0.7 m/s, biofilms maintained their original structure. In addition, the average roughness and thickness of each biofilm before and after exposure to different flow velocities were statistically the same (Table A.3). These observations that biofilm structure did not change during detachment experiments indicated that the biofilms grown from the groundwater were rigid enough to resist high shear stress caused by the high flow velocity. The rigid structure of biofilms may be due to the long time used for biofilm development, the low nutrient, and the
high hardness (1.63 mM Ca\(^{2+}\)) of the feed groundwater. Previous study also revealed a more rigid biofilm structure under reduced nutrient conditions. Calcium ions in the feed groundwater may strengthen biofilms structure by crosslinking the biofilm matrix, allowing better resistance to shear stress. Because the biofilms used in this study were resistant to a wide range of flow conditions (from 0 to 0.7 m/s), the effect of structural change during detachment experiments and flow simulation could be ignored.

**Figure 2.2** OCT images of 30-week biofilms under the average flow velocity of a) 0.1, b) 0.3, and c) 0.7 m/s. The yellow line is drawn manually and shows the boundary between the biofilm and water. All these images were captured when the biofilms were exposed to continuous flow with corresponding velocity. The flow exposing time was 30 minutes, and biofilms were imaged at the interval of 1 minute. The images of these biofilms under flow taken at 1st min and 30th min were shown here.
2.4.2 Adhesion experiments of *L. pneumophila* on biofilms grown on PVC coupons

*L. pneumophila* adhesion on biofilms with different roughness was experimentally measured for solutions containing from 3 to 300 mM ionic strength to determine whether electrostatic double layer compression or biofilm surface roughness control the adhesion. *L. pneumophila* adhesion on PVC surfaces and 2-week biofilms increased with ionic strength (Figure 2.3a). This observation with fluorescent microscopy was consistent with lower electrostatic repulsion between PVC surface and *L. pneumophila* cells based on less negative electrophoretic mobility values of the cells at higher ionic strength. The electrophoretic mobility of *L. pneumophila* cells was -1.90±0.09, -1.58±0.10, and -0.52±0.06 μm·V/(s·cm) (N=12) at 3, 10, and 100 mM, respectively (Figure A.2). At 300 mM, the adhesion on both PVC and the 2-week biofilm was lower than that at 100 mM. The observation that adhesion leveled off with further increases in ionic strength has already been reported for other colloidal particles. In contrast to the observation that *L. pneumophila* adhesion on PVC and 2-week biofilm surfaces is dependent on ionic strength, we found that on those biofilms older than 4 weeks with thickness from 20 to 32 μm (Table A.1), the Sherwood numbers for *L. pneumophila* were similar at ionic strengths from 3 to 300 mM (Figure 2.3a), indicating ionic strength did not control *L. pneumophila* adhesion on older biofilms. For example, on the 14-week biofilm, the Sherwood number values obtained at 3 mM, 10 mM, and 100 mM were statistically similar (t-test, α=0.05, p=0.9). *L. pneumophila* adhesion measured by CLSM was also independent of ionic strength (Figure 2.3b). In addition, Sherwood numbers obtained for the 14-week biofilm at 10 mM KCl using these two imaging methods were statistically similar (p=0.85). The observation that CLSM imaging gave the same results as fluorescence microscopy suggested that, under these testing conditions, *L. pneumophila* adhered to the biofilm surface.
instead of penetrating into the biofilm matrix. The Sherwood numbers measured for all cases were less than one, varying from 0.003±0.001 to 0.08±0.03, in agreement with previously reported values of Sherwood numbers from 0.004 to 0.29 for *E. coli* adhesion on bare and zeolite-coated aluminum alloy and stainless steel surfaces in 10-100 mM KNO₃ solution.⁴¹

**Figure 2.3** Sherwood numbers of *L. pneumophila* deposited on PVC and biofilm surfaces grown at different times as a function of ionic strength (KCl) examined in a) fluorescence microscope adhesion experiments and in b) CLSM adhesion experiments at pH 8.2-8.5 and at 25 °C. Adhered cells and deposited cells were quantified by fluorescence microscopy and CLSM, respectively.
While the adhesion of \textit{L. pneumophila} on older biofilms was independent of ionic strength, we found that the Sherwood numbers measured at both 3 mM and 100 mM correlated positively with the relative roughness coefficient (Figures 2.4 and A.3). Specifically, with biofilm relative roughness coefficient increasing from 0.30 ± 0.07 (14-week biofilm) to 0.76 ± 0.07 (4-week biofilm), Sherwood numbers increased from 0.03 ± 0.01 to 0.07 ± 0.02 at 3 mM. This observed higher adhesion on rougher surfaces could be explained by an enlarged surface area due to the surface roughness as reported previously.\textsuperscript{52} However, while the surface area enlargement parameter of the roughest biofilms was 1.5 times larger than that of the smoothest biofilms (3.2 for 4-week biofilms vs. 2.1 for 14-week biofilms), the adhesion of \textit{L. pneumophila} on the roughest biofilms was twice larger than that on the smoothest biofilms. Thus, other factors besides the enlarged surface area contributed to the higher adhesion on rough surfaces.

![Figure 2.4](image.png)

**Figure 2.4** Sherwood numbers of \textit{L. pneumophila} examined in fluorescence microscope adhesion experiments as a function of relative biofilm roughness coefficient at 3 mM.

Cell adhesion is controlled by surface interactions and hydrodynamics in flow conditions.\textsuperscript{25,53} As observed here, the increase in ionic strength and reduction in electrostatic repulsion did not
lead to higher adhesion on older biofilms. Previous study also reported that the local hydrodynamics near the surface overcome the repulsive DLVO interactions and make the roughness asperity act as attractive locations allowing the particles getting closer to the substrate surface.\textsuperscript{54} Therefore, effects of hydrodynamics on \textit{L. pneumophila} adhesion should be considered.

To explain how local hydrodynamic conditions created by surface roughness influences adhesion of particles with similar size and density as \textit{L. pneumophila} cells, we performed simulation of the flow above the biofilm surface and the movement of particles in the flow. This simplifying assumption will only allow an indirect and qualitative comparison of the experimental trend with simulation results.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.5.png}
\caption{Particle tracing simulation for a) a rough 4-week biofilm and b) a smooth 14-week biofilm at an average flow velocity of 0.007 m/s. c) Particles accumulated in the peak of one of the asperities in rough biofilm. d) Particles accumulated in the peak and the side facing flow in one aspertity in rough biofilm. Particle size is not drawn to scale. The horizontal length is 1 mm.}
\end{figure}
2.4.3 Hydrodynamics and particle tracing simulation for low flow velocity conditions used in adhesion experiments

The simulation results for flow velocity distribution and particle tracing above selected rough (4-week) and smooth (14-week) biofilm contours exposed to an average flow velocity of 0.007 m/s were obtained to determine the role of surface roughness on particle deposition. As shown in Figure 2.5a and 2.5b, particles adhered more on the rough surface compared with that on the smooth surface. The average values of deposition probability on ten rough and ten smooth biofilm surfaces were 0.13±0.03 and 0.06±0.01, respectively. Statistically higher particle adhesion (t-test, p=0.0002) obtained for rough surfaces compared with smooth surfaces suggested that the surface roughness enhanced particle deposition. On the rough biofilm surface (Figure 2.5a), most of the particles accumulated near the peak and on the side of the asperity that was facing the flow. On the smooth surface (Figure 2.5b), however, adhered particles were distributed more randomly along the biofilm surface.

Based on the particle capture theory,\textsuperscript{55} we propose that the direction change of streamline above the rough surface enhanced the interception of particles with the rough surface asperities, allowing additional particle adhesion. The distribution and shape of the streamline was highly dependent on the structure of the surface boundary. Specifically, along the rough surface, the direction of the velocity vectors changed significantly (Figure 2.5a). In contrast, along the smooth surface, the velocity vectors maintained their horizontal direction. When particles moved with the flow streamline and got closer to the asperity present on the rough surface, these particles could be directly blocked by this asperity or impact with this asperity by inertia (Figure 2.5c and 2.5d). This process was facilitated at the location where the streamline intercepted with roughness...
asperities or where flow direction changed dramatically, allowing more particles to accumulate at the peaks and the side of the asperity that was facing the flow. However, on the smooth surface, less particle interception was expected due to less variation of the streamline direction along the surface. Comparing the velocity distribution on both the rough and the smooth surfaces, a larger stagnant zone was observed surrounding asperities on the rough surface versus the smooth surface. In these zones, particles could slowly move along the asperities, allowing enhanced interception between particles and roughness asperities. On a smooth surface, by contrast, there is a low probability of particle interception with surface roughness asperities. In summary, the higher particle adhesion on rougher surfaces appeared to be due to the enhanced interception resulting from the local hydrodynamic conditions created by surface roughness.

2.4.4 Qualitative comparison of experimental results and simulation results

The results of \emph{L. pneumophila} adhesion experiments show that \emph{L. pneumophila} adhesion was enhanced on rougher biofilms. The simplified particle tracing simulation, for the first time, showed the detailed local flow profile and particle movement above complex biofilm profiles obtained by OCT. The simulation results revealed the enhanced particle interception on rough surfaces in agreement with the experimental results. While this simulation identified the roles of surface structure on adhesion, it may not exactly reflect the movement of \emph{L. pneumophila} in a real flow system, such as DWDS, due to the following limitations. 1) Particles used in simulation were sphere shaped, while \emph{L. pneumophila} cells are rod shaped. In our simulations, the micrometer scale difference was not considered due to the resolution of biofilm contours obtained from OCT technique. 2) For clearly showing the effect of surface roughness along the flow direction, we only
conducted 2-D simulations above the cross-section profile of biofilms. 2-D simulations were commonly used in previous studies on hydrodynamics simulations for biofilms. 56, 57 The possible particle diffusion and flow disturbances perpendicular to the main flow direction in 3-D space were not considered. 3) The simulation was conducted under a flow condition, including particle diffusion and convection. Under completely stagnant flow conditions in DWDS, particle diffusion will dominate the adhesion. Overall, although this simulation could not precisely represent the transport of L. pneumophila in real DWDS, it provided evidence of roughness enhancing particle adhesion by creating local hydrodynamics and supported the conclusions obtained from the adhesion experiments.

2.4.5 Detachment experiments of L. pneumophila from biofilms

Detachment of pre-adhered L. pneumophila from a selected rough biofilm and a smooth biofilm was experimentally determined at average flow velocities of 0.1, 0.3, and 0.7 m/s, which simulated the flow rate in DWDS. The ratios of cells retained on the biofilm to the total pre-adhered cells on the biofilm ($R_t$) as a function of time were determined. For both rough and smooth biofilms, $R_t$ dropped rapidly with time, then became stable after a few minutes. For example, when the smooth biofilm was subjected to an average flow velocity of 0.1 m/s, $R_t$ decreased from 1 to 0.42 in the first 6 min, then stopped decreasing over the next 24 min (Figure A.4). The time required to achieve 90% of maximal cell detachment ($T_{90}$) and the final ratio of the total detached cells to total pre-adhered cells ($D_{final}$) for different flow conditions were calculated (Table A.6). An increase in average flow velocity from 0.1 to 0.7 m/s led to higher detachment. For example, for the rough surface, $D_{final}$ of 45%, 53%, and 73% were obtained under average flow velocities of 0.1, 0.3, and
0.7 m/s, respectively, indicating that more cells detached under the higher average flow velocity. In addition, $T_{90}$ decreased from 9.8 minutes to 3.3 minutes when the average flow velocity increased from 0.1 to 0.7 m/s, revealing a faster detachment of *L. pneumophila* under the higher flow velocity. Higher shear stress caused by higher flow velocity was reported to lead to the increased cell detachment under increasing flow velocity.\textsuperscript{25, 34} Therefore, the observed dependence of *L. pneumophila* detachment with flow velocities was further explained using the simulation results of shear stress distribution in the flow chamber (Figure A.5).

As evidence from the OCT imaging and analysis, biofilms grown from groundwater used in this study had rigid structure resisting deformation when subjected to flow velocities up to 0.7 m/s. For this reason, biofilm deformation was not considered in the simulation for shear stress exerted by the water flow on the biofilm. According to the simulation results, when the average flow velocity increased from 0.1 to 0.7 m/s, the shear stress on both rough and smooth surfaces increased significantly. This increased shear stress with flow velocity has been shown to be responsible for the improved detachment rate of bacteria from glass surfaces.\textsuperscript{25, 34} In our study, the increased shear stress with increasing flow velocity also caused a 3 times faster *L. pneumophila* detachment from biofilms.

In addition to the observed detachment trend with flow velocity, detachment of *L. pneumophila* also depended on the biofilm roughness. Under the average flow velocities of 0.1 m/s and 0.3 m/s, higher detachment was observed from smooth biofilm surface compared to rough biofilm surface. Under 0.3 m/s average flow velocity, $T_{90}$ for the rough and smooth biofilm surface was 6.61 and 3.38 min, respectively, revealing a faster *L. pneumophila* detachment from the smooth biofilm surface. $D_{\text{final}}$ of 53% and 74% were obtained for the rough and the smooth biofilm surface, indicating that larger amounts of pre-adhered cells were detached from the smooth biofilm surface.
In contrast to the observation at lower flow velocities of 0.1 m/s and 0.3 m/s, under an average flow velocity of 0.7 m/s, similar detachment of *L. pneumophila* from both rough and smooth biofilms was observed. Specifically, 73% and 77% of pre-adhered cells detached from the rough and the smooth biofilm surfaces at the end of detachment experiments, respectively.

Previous modeling study reported that larger hydrodynamic force would be required to detach particles from a rougher surface compared to a smooth surface. Therefore, we compared the shear stress profiles exerted on the smooth and rough surfaces studied here. Compared with rough surface, the average flow velocities from 0.1 to 0.7 m/s exerted a more uniform shear stress distribution on the smooth surface. For example, under the average flow velocity of 0.3 m/s, on the rough surface (Figure A.5b), the highest shear stress was formed near the peak of each asperity (cyan, yellow, and red areas with shear stress > 6 Pa), while large low shear stress zones were formed underneath the peak (dark blue areas with shear stress < 2 Pa). On the smooth surface (Figure A.5e), shear stress on most of the area was >6 Pa. The larger low shear stress zones on the rough biofilm surface suggested that cells adhered in these zones were subjected to less shear stress penetration and therefore had a lower probability of detachment. On the smooth biofilm surface, however, the shear stress was distributed more uniformly, thus most of the biofilm surface was exposed to shear stress. Consequently, in contrast to the rough surface, more cells were expected to detach from the smooth biofilm surface. However, under the highest average flow velocity of 0.7 m/s used here, the high shear stress exerted on the biofilm may penetrate the biofilm causing detachment of biofilm surface layer, not just the pre-adhered cells. For this reason, high shear stress caused the equally high detachment of *L. pneumophila* from both smooth and rough biofilm surfaces.
In summary, this study identified that *L. pneumophila* adhesion was enhanced by biofilm roughness because of the increased interception between the flowing particles and the surface on rough biofilms. After *L. pneumophila* adhered to the biofilm, subsequent cell detachment was facilitated by high average flow velocity. Biofilm roughness could protect *L. pneumophila* from detachment by creating larger low shear stress zones. A summary of the study results is provided in Table A.7. These findings are relevant for pathogen control within premise plumbing. However, the *L. pneumophila* long-term colonization and release should be evaluated to shed light upon the fate and transport of pathogenic *L. pneumophila* in DWDS. The effect of practical conditions (e.g., temperature) and drinking water components (hardness, disinfectant, the presence of amoeba) need further investigation.

2.5 References


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CHAPTER 3: RESPONSE OF SIMULATED DRINKING WATER BIOFILM
MECHANICAL AND STRUCTURAL PROPERTIES TO LONG-TERM DISINFECTANT EXPOSURE

3.1 Abstract†

Mechanical and structural properties of biofilms influence the accumulation and release of pathogens in drinking water distribution systems (DWDS). Thus, understanding how long-term residual disinfectants exposure affects biofilm mechanical and structural properties is a necessary aspect for pathogen risk assessment and control. In this study, elastic modulus and structure of groundwater biofilms was monitored by atomic force microscopy (AFM) and optical coherence tomography (OCT) during three months of exposure to monochloramine or free chlorine. After the first month of disinfectant exposure, the mean stiffness of monochloramine or free chlorine treated biofilms was 4 to 9 times higher than those before treatment. Meanwhile, the biofilm thickness decreased from 120±8 \( \mu \text{m} \) to 93±6 -107±11 \( \mu \text{m} \). The increased surface stiffness and decreased biofilm thickness within the first month of disinfectant exposure was presumably due to the consumption of biomass. However, by the second to third month during disinfectant exposure, the biofilm mean stiffness showed 2 to 4-fold decrease while the biofilm thickness increased to 110±7 -129±8 \( \mu \text{m} \), suggesting that the biofilms adapted to disinfectant exposure. After three months of the disinfectant exposure process, the disinfected biofilms showed 2-5 times higher mean stiffness (as determined by AFM) and 6-13 fold higher ratios of protein over polysaccharide,

as determined by differential staining and confocal laser scanning microscopy (CLSM), than the non-disinfected groundwater biofilms. However, the disinfected biofilms and non-disinfected biofilms showed statistically similar thickness (t-test, p>0.05), suggesting that long-term disinfection may not significantly remove net biomass. This study showed how biofilm mechanical and structural properties vary in response to a complex DWDS environment, which will contribute to further research on the risk assessment and control of biofilm-associated-pathogens in DWDS.

3.2 Introduction

Biofilms in drinking water distribution systems (DWDS) can facilitate pathogen persistence and transmission\(^1\) by harboring pathogens\(^2\), supplying nutrients,\(^3\)-\(^7\) and protecting pathogens from disinfection.\(^8\),\(^9\) It is further reported that biofilms can capture or accumulate planktonic pathogens and then release these pathogens via the detached biofilm materials.\(^1\) This process (biofilms accumulating and releasing pathogens) can be highly influenced by biofilm structural and mechanical properties. For example, biofilm roughness was observed to control pathogen accumulation to biofilms by increasing the interception of pathogens with biofilms.\(^10\)-\(^13\) Biofilm elasticity and cohesiveness are shown to be essential for detachment of biofilms and biofilm-associated pathogens.\(^14\)-\(^16\) Therefore, comprehensive understanding of the mechanical and structural properties for drinking water biofilms will provide information to predict, assess, and aid in controlling the risk of pathogens associated with DWDS biofilms.

A disinfectant residual is required in most drinking waters by the U.S. Environmental Protection Agency (EPA). Of particular interest here is that disinfectant residuals may influence the biofilm mechanical and structural properties through biomass loss and change in biofilm
chemical composition. Thinner and rougher *Pseudomonas aeruginosa* biofilms were observed after a relatively short term (1-6 days) of continuous exposure to free chlorine stream.\(^{17}\) The cohesiveness of multi-species drinking water biofilms did not significantly change after 60 mins of exposure to quiescent free chlorine solution.\(^{18}\) Longer disinfectant exposure (8 weeks) was also reported to lead to a reduction in groundwater biofilm thickness.\(^{19}\) However, it is unknown how longer term (i.e., normal operational) disinfectant exposure may influence mechanical and structural properties other than thickness. In addition to disinfectant exposure, hydrodynamic shear stress is known to influence biofilm mechanical and structural properties.\(^{18, 20-25}\) For example, biofilms developed under high shear stress up to 10 Pa were shown to be cohesively stronger.\(^{15, 21}\) Reduction of biofilm thickness was observed under a continuous exposure to shear stress up to 0.9 Pa.\(^{21, 22}\) During disinfectant exposure, shear can accelerate biofilm-disinfectant reaction by enhancing mass transfer of disinfectant into the biofilms,\(^{26}\) presumably leading to significant biofilm property variation. However, the combined effect of disinfectant exposure and shear stress on properties of biofilm grown under low nutrient conditions over a longer time appears to be unreported.

To fill these research gaps, mechanical and structural properties of simulated drinking water biofilms were monitored during three months of disinfectant exposures. Monochloramine and free chlorine are the two most commonly used disinfectants in DWDS and were separately used to treat groundwater-grown biofilms. Both shear and quiescent conditions were explored during disinfectant exposure to simulate dynamic and stagnant zones in DWDS. In this study, we measured biofilm elastic modulus with atomic force microscopy (AFM) and biofilm structure (thickness and roughness) with optical coherence tomography (OCT) to determine the role of disinfectant exposure, shear conditions, and exposure duration time on biofilm mechanical and
structural properties. The results of this study show how biofilm mechanical and structural properties vary in response to a complex DWDS environment and contribute to further research on the risk assessment and control of biofilm-associated-pathogens in DWDS.

3.3 Materials and Methods

3.3.1 Biofilm preparation and disinfectant exposure assay

A groundwater source for drinking water in Urbana-Champaign, IL was selected for growing biofilms on PVC coupons (RD 128-PVC, BioSurface Technologies Corporation, Bozeman, MT) in CDC reactors (CBR 90-2, BioSurface Technologies Corporation, Bozeman, MT), as described previously.\textsuperscript{11,12} This groundwater source mainly contained 1.6 mM Ca\textsuperscript{2+}, 1.2 mM Mg\textsuperscript{2+}, and 1.0 mM Na\textsuperscript{+}. Continuous stirring at 125 RPM in the CDC reactors created a shear condition with a Reynolds (Re) number of 2384. The groundwater biofilms were developed in CDC reactors for one year and then distributed in six reactors for the subsequent biofilm disinfectant exposure.

In the biofilm disinfection step, these one-year-old biofilms were exposed to either free chlorine or monochloramine for 3 months. Specifically, groundwater containing 4 mg Cl\textsubscript{2}/L of monochloramine or free chlorine were continuously introduced to the biofilm reactors in either a stirred or non-stirred condition (Figure 3.1). Reactors 1 and 4 were continuously exposed to groundwater containing freshly prepared monochloramine, while Reactors 2 and 5 were similarly exposed to freshly prepared free chlorine. Reactors 3 and 6 were exposed only to disinfectant-free groundwater and were used as controls. Reactors 1-3 were stirred at 125 RPM to simulate pipe flow conditions, while Reactors 4-6 were unstirred to simulate stagnant conditions. Both stirred
and non-stirred conditions were used because the shear stress caused by stirring could influence the biofilm mechanical and structural properties.\textsuperscript{18,20-25} In a real DWDS, both quiescent and shear conditions are likely to occur and affect biofilm structure. The six reactors were operated three months at 4 mg Cl\textsubscript{2}/L of total disinfectant, which is the maximal residual disinfectant concentration in DWDS required by the EPA. The feed disinfectant solutions were prepared and replenished every other day.

\textbf{Figure 3.1} The experimental setup of disinfectant exposure assay for biofilms. GW: groundwater.
3.3.2 Biofilm elastic modulus determination by AFM indentation test

3.3.2.1 AFM probe preparation

The indentation measurements were conducted with a silica sphere (with a diameter of 20 µm) glued to a tipless cantilever (calibrated with a normal spring constant of 0.6-1.2 N/m, Mikromasch, Lady's Island, SC). Similar to previous studies using spherical probes ranging in size from 10 µm to 50 µm to detect mechanical properties of polymers, cells, and biofilm,\textsuperscript{27-29} spherical beads with a diameter of 20 µm, instead of a sharp tip (tip radius <10 nm), were chosen to create larger contact areas and small contact pressures between the probe and the biofilm. A 20 µm diameter spherical probe can lead to a projected area of \(\sim 54 \ \mu m^2\) on a biofilm surface at the maximal indentation depth of 5 µm, based on our measurements (e.g., a contact radius of \(\sim 4.1 \ \mu m\) is obtained at a load of 52 nN for a rigid silica sphere and a flat biofilm of \(\sim 5kPa\), according to the Hertz model\textsuperscript{30}). Therefore, the 20 µm diameter spherical probe provided better representation of the biofilm elastic modulus at mesoscale compared with the submicron scale measurement conducted by a AFM sharp tip. More details of building a spherical probe were described previously\textsuperscript{31} and are also in the appendix.

3.3.2.2 Indentation test data collection and analysis

The change in biofilm elastic modulus (Young’s modulus) during the long-term disinfection process was estimated by indentation tests conducted on the same biofilm coupons from each
reactor every month. The test biofilm coupons were carefully removed from the reactors for the indentation tests, after which they were immediately returned to the biofilm reactors. The biofilms were kept in groundwater during all the transit and experimental process to avoid dehydration. All of the indentation measurements were performed in groundwater filtered through 0.22 \( \mu \text{m} \) cellulose membrane. The test biofilm coupons were gently rinsed with filtered groundwater three times before being subjected to indentation tests. The contact mode of an MFP-3D AFM (Asylum Research, Santa Barbara, CA) was used for all indentation tests. Before indentation all AFM probes were calibrated on a bare glass surface both in air and in liquid to obtain the cantilever deflection sensitivity for force calculation.27

Following the probe calibration, the indentation tests were carried out with a probe approaching the velocity of 2 \( \mu \text{m/s} \). The indentation force was measured as a function of indentation depth (Figure 3.2). The probe was indented into the biofilms with maximal indentation depth of 5\( \mu \text{m} \). The indentation depths were limited within 10% of the total biofilm thickness to avoid the interference of the PVC substrate.32,33 Prior to the probe contacting and indenting into the biofilms, surface forces, including electrical double layer, hydration, and steric interactions, between the probe and the biofilm can lead to a weak repulsion. The maximal range of the surface interaction was \(-100 \text{ nm}\) based on force measurements and its distinguishable force-law, thus a deconvolution of surface force and indentation force was possible in our measurements. After the probe overcame this weak surface repulsion, the probe penetrated (Indented) into the biofilms at a certain depth dependent on the applied force (50-500 nN) and biofilm mechanical properties. In some indentation measurements, a bilayer biofilm structure was revealed along the indentation depth. The indentation curve for the biofilm outer layer has a lower slope compared to the biofilm inner layer (Figure 3.2), suggesting that the Young’s modulus of the biofilm outer layer was
reproducibly smaller than that of the biofilm inner layer. AFM indentation tests were also used to characterize the bilayer structure of other soft materials, such as cells.\textsuperscript{27} The thickness of the outer layer was determined at the change of slope in the indentation curve (Figure 3.2), and the Young’s modulus ($E$) of the shell layer was determined by fitting the curve to the Hertz model,\textsuperscript{27} based on Equation 1:

$$F = \frac{4}{3} \times \frac{E}{1 - \nu^2} \times \sqrt{r} \times \delta^3$$  \hspace{1cm} (1)

Where $F$ is the force applied by the AFM probe to deform the biofilm surface, $\nu$ is the biofilm Poisson’s ratio (assumed to be $0.3^{14}$), $r$ is the radius of the AFM probe, and $\delta$ is the probe indentation depth. The MFP-3D AFM software was used to conduct the indentation curve fitting using the Hertz model (fitting results have reduced chi-square values close to 1). The outer layer Young’s modulus was determined in this study because the outer layer directly exposed to drinking water phase is expected to be more relevant to pathogen accumulation and release processes, while the biofilm inner layer was not characterized here due to the possible interference of PVC substrate.\textsuperscript{32}
Indentation test principle. A sphere probe with the diameter of 20 µm was used in the indentation measurement. When the probe was indented into the biofilm surface, the deflection of the probe cantilever was monitored, and the applied force was calculated with the measured vertical deflection and the spring constant of the cantilever. The force as a function of indentation depth was then plotted, and the biofilm outer layer Young’s modulus and thickness was determined accordingly.

Indentation measurements were repeated at 20-30 randomly selected locations in each biofilm sample. At each location, indentation tests were conducted at different applied loads ranging from 50-500 nN. At each applied load, the indentation tests were repeated 2-5 times. In total, 120-450 indentation tests were conducted on each biofilm sample. Due to biofilm heterogeneity, the distribution of the Young’s modulus values obtained from these randomly selected locations on each biofilm was analyzed to characterize the change in biofilm stiffness within a disinfectant exposure time period. Kolmogorov-Smirnov tests were used to compare the Young’s modulus distributions obtained for different biofilms. In addition, all the measured values of Young’s modulus for each sample were divided into four groups: very soft group with $E<5$ kPa, soft group with 5 kPa<$E<20$ kPa, hard group with 20 kPa<$E<100$ kPa, and very hard group with 100 kPa >$E$. For each biofilm sample, the percentage of each group in all measured $E$ values was determined.
3.3.3 Biofilm structure determination by OCT

The roughness and thickness of the biofilms were determined by the optical coherence tomography (OCT) technique described previously.12, 34 In this study, a custom built 1300 nm based spectral domain OCT system imaged biofilm cross sections of 3.1 mm transverse by 2.1 mm in depth with an axial resolution of 4.2 µm and a transverse resolution of 3.9 µm.35 To monitor the biofilm structural change, the biofilms collected every week for the first month and every two weeks for the second and third month were subjected to OCT imaging immediately after being removed from the reactor. A drop of groundwater was added on each biofilm coupon during OCT measurements to maintain the hydrated condition for biofilms. A volumetric scan consisting of two hundred images was taken in two separate locations from each sample. The average thickness and roughness were calculated from twenty randomly selected images for the biofilms in each reactor. ImageJ (http://imagej.nih.gov/ij/) was used to eliminate the background of these images before further analysis. Biofilm mean thickness and roughness were determined by analyzing grey scale gradient with automatic thresholding using the MATLAB program developed by Derlon et al.36

3.3.4 Biofilm composition determination by confocal laser scanning microscopy (CLSM)

To explore the possible connection between biofilm elasticity and biofilm composition, the composition of three months disinfected biofilms and control biofilms were determined by CLSM
Biofilms are mainly composed of bacteria cells and extracellular polymeric substances (EPS). The biofilm mechanical properties are highly dependent on EPS. To determine the amount of each component in EPS, the fluorescent dyes of Sypro Orange and a mixture of ConA Alexa 633 and WGA Alexa 633 (Thermo Fisher Scientific Inc., Waltham, MA) were used to stain biofilm protein and polysaccharide, respectively. Then the image of each component was scanned by CLSM, as described previously. Briefly, the stained protein was scanned at the excitation wavelength of 470 nm and recorded at the emission wavelength of 570 nm. The stained polysaccharide was scanned at the excitation wavelength of 633 nm and recorded at the emission wavelength of 647 nm. A series of horizontal sections of protein and polysaccharide in the biofilms were imaged at an interval of 0.37 µm along the thickness of the biofilms. For each biofilm sample, 6-7 different locations with the size up to 720×720 µm were selected for CLSM imaging. To obtain the composition of the biofilms, the CLSM images of the biofilms were further analyzed using COMSTAT. COMSTAT recognized the volume of protein and polysaccharide, respectively, by stacking each horizontal section image. The ratio of protein/polysaccharide was then determined accordingly. In this study, the volume of protein and polysaccharide determined by CLSM was not used to compare biofilm composition due to the heterogeneity in biofilm thickness and the possible diminishing of fluorescence signal along biofilm depth. Instead, the ratios of protein over polysaccharide in biofilm EPS under different conditions were compared, because these ratios reflected the change in biofilm EPS composition.
3.4 Results

3.4.1 Biofilm elastic modulus monitored by AFM at different disinfectant exposure and shear conditions

To compare the biofilm mechanical properties after three months of exposure to different disinfectants and hydrodynamics conditions, frequency distributions of the measured Young’s moduli ($E$) for the biofilms from different reactors were obtained. Compared to the $E$ values of non-disinfected groundwater biofilms (Reactors 3 and 6) (Figure 3.3a and 3.3b), the $E$ values of free chlorine (Figure 3.3c and 3.3d) and monochloramine treated biofilms (Figure 3.3e and 3.3f) clustered in a higher range (Kolmogorov-Smirnov test, $p<0.05$). For example, under the non-stirring (shear-free) condition (Figure 3.3b, d, and f), the maximal $E$ frequency for the groundwater biofilms was located in the lower range of 0-2 kPa; while for the free chlorine or monochloramine treated biofilms, the maximal $E$ frequency was located in the higher range of 2-4 kPa. $E$ distributing in the higher ranges indicated that exposure to free chlorine and monochloramine increased the biofilm Young’s modulus or stiffness. In addition, the $E$ distributions under the stirring (shear) and non-stirring (shear-free) conditions were compared. For the groundwater biofilms and free chlorine treated biofilms (Figure 3.3a vs. Figure 3.3b, Figure 3.3c vs. Figure 3.3d), the $E$ distributions under the stirring and non-stirring conditions were statistically the same (Kolmogorov-Smirnov test $p>0.05$), indicating that moderate hydrodynamic shear did not significantly alter the biofilm Young’s modulus after three months of disinfectant-free groundwater or free chlorine exposure. Conversely, the monochloramine treated biofilms indicated that a shear condition during monochloramine exposure increased the biofilm elastic modulus.
(Figure 3.3e vs. Figure 3.3f), given the statistically different $E$ distributions (Kolmogorov-Smirnov test, $p=0.04$). In summary, both free chlorine and monochloramine treatment increased the Young’s modulus $E$ or stiffness of the biofilms, while the shear condition increased biofilm stiffness only under the monochloramine exposure condition.

**Figure 3.3** The frequency distribution of $E$ for biofilm after 3 months exposure of groundwater a) with and b) without stirring, free chlorine c) with and d) without stirring, monochloramine e) with

- **a)** Groundwater Stirring
  - Mean = 3.27

- **b)** Groundwater No Stirring
  - Mean = 1.93

- **c)** Free chlorine Stirring
  - Mean = 8.66

- **d)** Free chlorine No Stirring
  - Mean = 7.53

- **e)** Monochloramine Stirring
  - Mean = 17.65

- **f)** Monochloramine No Stirring
  - Mean = 9.86

$E$ (kPa)
and f) without stirring, respectively. Y-axis represents the frequency of occurrences of $E$ values with each interval size of 5 kPa.

In addition to examining the biofilm Young’s modulus under different disinfectant exposure and shear conditions, the change in the Young’s modulus over disinfectant exposure time was also determined (Figure 3.4). Before disinfection treatment, all the measured Young’s modulus values belonged to either the very soft or the soft group ($E$ values smaller than 20 kPa). However, after one month of monochloramine treatment, the fraction of soft and very soft biofilms in the measured biofilms was reduced to 18%. Most of these biofilms were hard (20 kPa<$E<$100 kPa) or very hard ($E>$100 kPa). The mean value of $E$ after one month of monochloramine disinfection was eight times higher than that before treatment (42 kPa vs. 5 kPa). The increased $E$ values suggested that the biofilm stiffness was significantly increased after monochloramine exposure. However, after two or three months of exposure to monochloramine, soft and very soft biofilms again dominated the overall biofilm behavior. The fraction of soft and very soft biofilms in the total measured biofilms increased to 68% and 85% at the second and third month, respectively. The $E$ values at the second and third month were statistically similar (t-test, $p=0.73$) and the mean $E$ values were twice lower than that for the first month, suggesting that the biofilm stiffness decreased in the second month and then stabilized by the third month. The observation that $E$ increased in the first month of disinfectant treatment and then decreased in the following months was also observed for other disinfection treatment conditions (Figure B1a, Figure B2a, and Figure B3a). Thus, the biofilm stiffness increased after one month of disinfectant exposure, but it decreased and became stable with longer disinfection treatment (2 or 3 months).
Figure 3.4 a) The percentage stacked bar for the Young modulus and b) the outer layer thickness of biofilms during the three months of monochloramine treatment under stirring conditions. The red line in Figure a) shows the mean value of $E$ at each time point. The percentage stacked bars and outer layer thicknesses of biofilms during other treatment conditions are shown in Figure B1 (free chlorine treatment under stirring condition), Figure B2 (monochloramine treatment under non-stirring condition), and Figure B3 (free chlorine treatment under non-stirring condition).
The above trend for the Young’s modulus change over time was the reverse of the trend in average biofilm outer layer thickness (Figure 3.4, Figure B1, Figure B2, and Figure B3). As shown in Figure 3.4, the highest biofilm Young’s modulus (Figure 3.4a) and lowest biofilm outer layer thickness (Figure 3.4b) was observed at the first month during exposure to monochloramine with shear conditions. Similar observation of the highest $E$ corresponding to the lowest average biofilm outer layer thickness was also found for the biofilms under other disinfectant exposure and shear conditions (Figure B1, Figure B2, and Figure B3).

3.4.2 Biofilm structure monitored by OCT at different disinfectant exposure and shear conditions

The average thickness of biofilms after three months of exposure to different disinfectant and shear conditions was estimated using OCT (Figure B4a). There was no significant difference between biofilms with or without disinfectant exposure. Specifically, under shear conditions, all the monochloramine treated, free chlorine treated, and non-disinfected (control) groundwater biofilms had statistically similar thicknesses (129±8 µm, 127±19 µm, and 123±18 µm, respectively; t-test, $p>0.05$). However, under shear-free conditions, although the free chlorine treated and groundwater biofilms still had statistically similar thicknesses (117±6 µm vs. 118±10 µm, t-test, $p=0.75$), the monochloramine treated biofilms had a slightly lower biofilm thickness of 110±7 µm (t-test, both $p<0.05$). The similarity of thicknesses for the disinfected and non-disinfected biofilms suggested that three months of disinfection did not significantly reduce biofilm thickness. In addition, no apparent biofilm thickness change was measured under different hydrodynamic conditions for free chlorine treated biofilms and non-disinfected biofilms (t-test, both $p>0.05$). Nonetheless, the thicknesses of monochloramine treated biofilms under the shear condition were higher than those
under the shear-free condition (t-test, p=2.5×10⁻⁸). Overall, however, monochloramine and free chlorine treated biofilms did not show substantially lower thickness than the non-treated biofilms.

In addition to comparing the biofilm thickness under different disinfectant exposure and shear conditions, the change in biofilm average thickness over the three months of disinfectant exposure was also monitored (Table S1). A decrease in thickness under monochloramine treatment over the first five weeks of disinfection was observed (t-test, p<0.05), followed by an increase in thickness over the next eight weeks (t-test, p<0.05). The lowest biofilm thicknesses were observed at the fifth week for the monochloramine treated biofilm under the shear (105±6 µm) and the shear-free conditions (93±6 µm). For the free chlorine treated biofilm, the thinnest biofilm thickness was observed at the fourth week, with thickness values of 107±11 µm and 103±8 µm under shear and shear-free conditions, respectively. In the following weeks (fifth to thirteenth week), the thickness increased and then recovered to the initial thickness at the end of disinfectant exposure (biofilm thickness at week 0 vs. biofilm thickness at week 13, t-test, p>0.05). While the monochloramine and free chlorine exposure showed the lowest thickness at around month 1 (fourth or fifth week), the non-disinfected groundwater biofilms did not show significant change in thickness over the three months study, under either shear or shear-free conditions (t-test, p>0.05). Therefore, biofilm thickness decreased during a short term of disinfectant exposure (roughly one month), but the thickness increased over the rest of the disinfectant exposure period.

The biofilm roughness from each reactor was compared after three months of disinfectant exposure (Figure B4b). Under the same hydrodynamic conditions (shear or shear-free), the monochloramine treated biofilms showed statistically lower roughness than free chlorine treated biofilms (t-test, p<0.05). In addition, with the same disinfectant treatment, the biofilm roughness under the shear condition was significantly lower than that under the shear-free condition. For
example, the roughness for the monochloramine treated biofilms was 0.15±0.03 and 0.36±0.04 under the shear and the shear-free conditions (t-test, p=1.6×10⁻²⁰), respectively (Figure 3.5). Therefore, for the biofilms exposed to the same disinfectant, the shear condition led to significantly lower biofilm roughness than the shear-free condition.

**Figure 3.5** OCT images of monochloramine treated, free chlorine treated, and groundwater biofilms under shear condition.

The change of biofilm roughness over the three months of disinfectant exposure was also monitored (Table S2). Under the shear condition, roughness of monochloramine treated biofilms showed a 1.7-fold decrease in the first four weeks, and then remained constant in the following nine weeks. The roughness of non-disinfected groundwater biofilms also decreased 1.3-fold over the three months under the same shear condition. However, the free chlorine treated biofilms kept the statistically similar roughness before and after the three months of disinfection (0.25±0.02 vs. 0.26±0.05, t-test, p=0.25). Under the shear-free condition, roughness of all monochloramine treated, free chlorine treated, and non-disinfected biofilms kept increasing during the three-month experiment. For example, the biofilm roughness increased 1.4 times during monochloramine
exposure. In summary, under the shear condition, the biofilm roughness was reduced or did not change by the three-month experiment. In contrast, with stagnant conditions, the roughness increased for all examined treatment conditions.

3.4.3 Biofilm composition after long-term disinfection determined by CLSM

The ratio of protein over polysaccharide in biofilm EPS under different conditions was determined by CLSM after the three months of disinfectant exposure. For the biofilms without any disinfectant exposure, the ratios of protein/polysaccharide were 1.2±0.46 and 0.92±0.34 under shear and shear-free conditions, respectively. However, with the free chlorine exposure, the biofilms had protein/polysaccharide ratios of 6.29±3.19 and 6.66±3.58, significantly higher than those of groundwater biofilms (t-test, p<0.05). With monochloramine exposure, these protein/polysaccharide ratios were further increased (t-test, p<0.05), being 13.09±1.89 and 8.28±2.47, respectively. These higher protein/polysaccharide ratios after disinfectant exposure suggested that free chlorine and monochloramine either directly consumed more polysaccharide than protein in biofilm EPS and/or stimulated changes in the biofilm community that expressed the different ratios.

3.5 Discussion

In contrast to previous studies focusing on short-term exposure of biofilms to disinfectants,17, 18 our three-month disinfectant exposure study revealed the dynamics of biofilm structural and mechanical properties. Specifically, the least biofilm thickness and the highest stiffness were
observed after one month of disinfectant exposure, but these properties recovered after three months. To our best knowledge, this is the first report revealing how biofilm properties changed during a long-term disinfection. In the first month of disinfectant exposure, a decrease of 13-27 µm in overall biofilm thickness was observed, suggesting biomass consumption by disinfectant. In addition, an increase from 4 to 9 times in the biofilm stiffness together with reduction in the biofilm outer layer thickness in the first month may be attributed to the consumption of biofilm EPS by disinfectant in the outer layer and the lack of EPS production by the inactivated bacteria cells near the outer layer. However, with longer disinfectant exposure, the biofilms may adapt themselves to the disinfectant (e.g., by adjusting their microbial community\textsuperscript{19, 41}) and produce EPS again to replenish the outer layer, consistent with the observed increase (14-35 µm) of the biofilm thickness and decrease (2-fold to 4-fold) of the biofilm stiffness. Thus, although short-term disinfection can lead to thinner and stiffer biofilms, biofilms could recover with long-term disinfectant exposure, a condition relevant for DWDS.

The long-term disinfectant exposure did not lead to a significant difference of biofilm thickness between the disinfected and non-disinfected biofilms, but higher stiffness was observed for disinfected biofilms compared with non-disinfected biofilms. The thickness between disinfected and non-disinfected biofilms was similar presumably because the biofilms became resistant to disinfectant under a long-term disinfection. Specifically, certain microorganisms in biofilms could become more resistant to disinfection.\textsuperscript{42} Also, disinfectant exposure could generate selective pressure to certain microbial populations in biofilms.\textsuperscript{43, 44} Hence, after three months of disinfection, neither monochloramine nor free chlorine treated biofilms showed obvious differences from the non-disinfected groundwater biofilms. Unlike biofilm thickness, the biofilm stiffness of the disinfected biofilms were 3-5 times higher than that of the non-disinfected biofilms. This higher
biofilm stiffness was observed together with higher ratios of protein over polysaccharide, suggesting that the higher stiffness of the biofilms after disinfection exposure may be due to the higher fraction of EPS protein. A previous study also suggested that protein-rich regions on the Lactobacillus johnsonii bacteria surface were stiffer than that of polysaccharide-rich regions.45 Because stiffer biofilms may be more stable against shear stress,16 less detachment and release of biofilm-associated pathogens in DWDS would be expected. Therefore, although long-term disinfection could not significantly remove biofilms, less detachment of stiffened biofilms is expected.

Shear conditions during disinfectant exposure can also influence biofilm structural and mechanical properties. Compared to biofilms treated under shear-free conditions, the biofilms exposed to shear conditions were smoother due to the biofilm erosion caused by shear stress.14 These smoother biofilms were expected to be more resistant to shear force and detachment.46 In addition, shear stress during monochloramine exposure caused stiffer biofilms. A previous numerical modeling study of the biofilm mechanical behavior showed that shear stress compressed and reduced the voids inside biofilms, thus leading to more compact and stiffer biofilms.47 However, no internal voids or channels were observed in this study under the resolution of the OCT imaging system used. Other studies revealed the enhanced disinfectant mass transfer by shear,48, 49 which may also cause the enhanced disinfection reaction, and thus an increase in biofilm stiffness under shear stress. However, the stiffness of free chlorine treated biofilms under shear and shear-free conditions did not show any difference, suggesting that other factors, such as limited penetration of chlorine,50 may control the mechanical properties of these biofilms.

In this study, the evolution of the biofilm Young’s modulus during three months of exposure to disinfectants was characterized by AFM micro-indentation. A wide range of values for the
Young’s modulus has been reported in previous studies due to the use of diverse methods and biofilms.\textsuperscript{51-60} Most of the studies that used AFM nano- or micro-indentation to determine the Young’s modulus focused on single culture biofilms and revealed a biofilm Young’s modulus ranging from 0.1 kPa to 316 kPa.\textsuperscript{51, 58, 59, 61} Only one study applied nano-indentation to drinking water biofilms and reported a biofilm Young’s modulus greater than 200 kPa.\textsuperscript{51} In our study, the lowest biofilm Young’s modulus was found to be 0.3 kPa (for non-disinfected biofilms), and the highest biofilm Young’s modulus was 179 kPa (for monochloramine treated biofilms). These values are within the range of previously reported biofilm Young’s modulus.

Our study applied AFM micro-indentation on multi-culture biofilms, providing a promising and non-destructive way to determine micro scale drinking water biofilm mechanical properties in a liquid environment. However, AFM indentation has some limitations in determining the mechanical behavior of biofilms, including that 1) only the elastic modulus of the outer layer can be quantitatively determined. Although a stiffer biofilm inner layer was revealed in the indentation curves and previous studies,\textsuperscript{47, 62} it was not characterized by indentation owing to relevant substrate effects; 2) the elastic modulus could only be measured along the biofilm depth but not in the lateral direction. However, a certain anisotropy of the biofilm is expected; 3) the shear modulus is also essential to determine biofilm resistance under shear stress and it cannot be determined from AFM micro-indentation. To overcome these limitations, other techniques could be combined with AFM indentation. For example, rheometers and tensile tests could be used to measure the mesoscale biofilm main body mechanical properties,\textsuperscript{53, 56, 60, 63-65} while AFM sharp tips could be used to abrade biofilm and explore biofilm mechanical strength along the horizontal direction.\textsuperscript{15, 18} Future efforts will focus on taking advantage of multiple techniques to comprehensively characterize the bulk mechanical behavior of biofilms.
The results of this study on biofilm mechanical and structural properties under long-term disinfectant exposure provide insights on pathogen transmission prediction and control in DWDS. Specifically, the biofilm elastic modulus and structure measurement results suggested that 1) more rigid biofilms after long-term disinfectant exposure may be more resistant to detachment, which can thus reduce the release of biofilm-associated pathogens; 2) shear stress in DWDS could help to maintain relatively smoother biofilms, on which less pathogen accumulation and biofilm detachment is expected; 3) disinfectant exposure in one month would have the best effect on increasing biofilm stiffness and reducing biofilm thickness, while longer time disinfection will lead to a decrease of biofilm stiffness and a recovery of biofilm thickness. Accordingly, risk assessment on DWDS pathogens could incorporate the information of biofilm mechanical and structural properties to precisely evaluate the biofilm-associated pathogen release level under disinfectant exposure conditions. In the next step, the connection between pathogen transmission and long-term disinfected biofilms needs to be further explored, and a mathematical model will be built to estimate the risk of pathogens in DWDS with disinfectant exposure.

3.6 References


CHAPTER 4: EFFECT OF DISINFECTANT EXPOSURE ON LEGIONELLA PNEUMOPHILA ASSOCIATED WITH SIMULATED DRINKING WATER BIOFILMS: RELEASE, INACTIVATION, AND INFECTIVITY

4.1 Abstract‡

Legionella pneumophila, the most commonly identified causative agent in drinking water associated disease outbreaks, can be harbored by and released from the drinking water biofilms. However, how the disinfectant residual in drinking water influences the release and activity of biofilm-associated L. pneumophila has not been well understood. In this study, the release of biofilm-associated L. pneumophila under flowing water containing disinfectant as well as the inactivation and infectivity of these released L. pneumophila were examined by qPCR, plate counting, and amoeba infectivity assay. The biofilms used in this study were exposed to disinfectant (pre-disinfected biofilms) or not exposed to disinfectants (non-disinfected biofilms) for six months to mimic the real drinking water biofilms developed under different disinfectant exposure conditions. The L. pneumophila release kinetics from pre-disinfected and non-disinfected biofilms did not show statistical difference (One-way ANOVA, p>0.05). However, inactivation of the L. pneumophila released from pre-disinfected biofilms was 1-2 times higher than that from non-disinfected biofilms. Meanwhile, L. pneumophila released from pre-disinfected biofilms showed 2 to 9 times lower infectivity than that from non-disinfected biofilms. The inactivation and infectivity results suggest that non-disinfected biofilms provided better protection for L. pneumophila from disinfection, likely due to the detachment of a larger amount of biofilm.

‡ This chapter is in preparation for a journal publication: Yun Shen, Conghui Huang, Jie Lin, Wenjing Wu, Nicholas Ashbolt, Wen-Tso Liu, and Thanh H. Nguyen. Effect of disinfectant exposure on Legionella pneumophila associated with simulated drinking water biofilms: release, inactivation, and infectivity
materials (determined by 16S rRNA qPCR analysis) surrounding the released \textit{L. pneumophila}. Our study highlights the interaction between disinfectant residual, biofilms, and \textit{L. pneumophila}, which provides guidelines to assess and control the potential health risks of \textit{L. pneumophila}.

4.2 Introduction

\textit{Legionella pneumophila} (\textit{L. pneumophila}), causative agent of Legionnaires’ disease, is the most commonly reported pathogen in drinking water leading to disease outbreaks in United States.\textsuperscript{1-4} From 2011 to 2012, drinking water \textit{L. pneumophila} led to 21 disease outbreaks, contributing to 66\% of the total reported disease outbreaks associated with drinking water in United States.\textsuperscript{4} Although disinfectant residual is required in most drinking waters by U.S. Environmental Protection Agency (USEPA), the \textit{L. pneumophila} can be more resistant to disinfectants when associated with drinking water biofilms.\textsuperscript{5-11} Biofilms in drinking water distribution or premise plumbing systems accumulate \textit{L. pneumophila} by capturing \textit{L. pneumophila} and providing nutrients to \textit{L. pneumophila}.\textsuperscript{10, 12} Some of these biofilm-associated \textit{L. pneumophila} cells can be protected from disinfection because biofilms could consume disinfectant and reduce disinfectant transfer to \textit{L. pneumophila}.\textsuperscript{7, 13} Subsequently, these \textit{L. pneumophila} cells can be released from biofilms with sloughing off biofilms and finally reach consumers with the drinking water flow. Therefore, biofilms protection and disinfectant inactivation are the two key factors determining the health risk of \textit{L. pneumophila} released from drinking water systems. Mechanistic understanding the interactions between biofilms, disinfectants, and \textit{L. pneumophila} is critical for developing \textit{L. pneumophila} control strategy.
The biofilm composition and disinfectant species were found to influence the disinfection efficacy of biofilm-associated *L. pneumophila* or other pathogens.\textsuperscript{14-20} The biofilms consist of mono-species *Microbacterium phyllosphaerae* or *L. pneumophila* were shown to be less tolerant to hydrogen peroxide or dendrimer exposure than the mixed-species biofilms.\textsuperscript{19, 20} In addition, monochloramine provided better disinfection efficiency on the bacteria in biofilms than free chlorine due to better diffusion of monochloramine into biofilms.\textsuperscript{17, 18} While most of these previous studies investigated the disinfection for biofilm-associated pathogens under quiescent conditions, disinfection of the biofilm-associated pathogens under drinking water flow conditions was largely ignored. Flow shear stress can continuously bring disinfectants to the biofilm surface and allow enhanced their mass transfer to the biofilms,\textsuperscript{21} thus may enhance the disinfection efficiency of biofilm-associated pathogens. However, the shear stress may have a counter effect to increase the risk of pathogen release from the biofilms.\textsuperscript{12} The disinfection and concurrent release of biofilm-associated pathogens under a continuous flow condition was not well explored previously. Moreover, in a real drinking water system, non-uniform exposure of biofilms to disinfectants may exist because of disinfectant consumption (by the biomass, natural organic matter, or pipe materials), altered temporal and spatial flow rate, and different water system design. The biofilms grown under different disinfectant exposure could have unique chemical compositions and mechanical properties, thus may protect biofilm-associated pathogens from disinfection to different extent.\textsuperscript{22-24} How the disinfectant exposure during biofilm development influences the subsequent biofilm-associated pathogen release and disinfection is unknown. In addition, previous studies showed that planktonic *L. pneumophila* cells were still able to infect their host, *Acanthamoeba castellanii*, 4 month after monochloramine treatment.\textsuperscript{25} However, the infectivity of released biofilm-associated pathogen with the presence of residual disinfectants was
underexplored. Therefore, a comprehensive study on the release, inactivation, and infectivity of the biofilm-associated pathogen under disinfectant exposure during a continuous flow is needed to understand the pathogen transmission in a real drinking water system.

Our study aims to understand the interactions between biofilms, disinfectants, and *L. pneumophila*, and to provide guidelines to assess the potential health risks of *L. pneumophila*. We firstly developed the biofilms from low-nutrient groundwater, under disinfectant-exposure or disinfectant-free conditions. The exposure of biofilms to disinfectants in biofilm development (i.e., pre-disinfected biofilms) simulated drinking water biofilms in real distribution systems, when residual disinfectants are well-maintained. Biofilms grown in groundwater without the presence of disinfectants (i.e., non-disinfected biofilms) was used to simulate the scenario that residual disinfectants are fully consumed. Next, to mimic the pathogen release process in drinking water systems, the water containing disinfectant continuously flew over the biofilms and released the biofilm-associated *L. pneumophila*. The released samples were then collected for the further analysis of *L. pneumophila* release kinetics, inactivation, and infectivity. The effect of pre-disinfecting biofilms and disinfectant species was then identified accordingly.

4.3 Materials and Methods

4.3.1 Biofilm preparation

Biofilms used in this study were developed from filtered groundwater, a source of drinking water in Urbana–Champaign, IL. This groundwater source has pH of 7.5 and hardness of 280 mg/L. To remove excessive mineral precipitates formed when the groundwater is exposure to air, this ground
water was filtered by green sand filtration. As described previously, this groundwater was continuously introduced into CDC reactors (CBR 90-2, BioSurface Technologies Corporation, Bozeman, MT) with continuous stirring at 125 rpm or Re of 2384 to develop biofilms on PVC coupons (RD 128-PVC, BioSurface Technologies Corporation, Bozeman, MT).

After one year of growing biofilms by groundwater, these biofilms were then distributed in three reactors for the subsequent six months of disinfectant exposure (Figure 4.1). Specifically, groundwater containing free chlorine or monochloramine (4 mg Cl₂/L) were continuously introduced to reactor 1 and 2 for six months, respectively. Reactor 3 was only exposed to the disinfectant-free groundwater. The reactor 1 and 2 were used to simulate the high disinfectant exposure conditions in DWDS, while the reactor 3 was used to simulate the situation when disinfectant was not sufficient. The feed disinfectant solutions were prepared and replenished every other day.

**Figure 4.1 Biofilm preparation and *L. pneumophila* release conditions**
4.3.2 Disinfection and release of biofilm-associated L. pneumophila

To simulate the association of pathogenic *L. pneumophila* to drinking water biofilms, *L. pneumophila* (ATCC 33152) was pre-adhered onto the monochloramine treated, free chlorine treated, and non-disinfected biofilms using a parallel plate flow chamber (FC 71, BioSurface Technologies Corporation, MT), as described previously. Briefly, groundwater containing 1–5 × 10⁷ cells/mL of *L. pneumophila* was pumped into the flow chamber at an average flow velocity of 0.007 m/s for half an hour. These adhered cells were then allowed to incorporate into the biofilms under quiescent flow conditions for two days before releasing from biofilms.

The process of *L. pneumophila* release from biofilms by the flowing drinking water containing or lacking disinfectant was simulated in the flow chamber to resemble the situations when residual disinfectant concentrations varies. Briefly, the groundwater containing disinfectant or groundwater free of disinfectant was introduced into the flow chamber at a high flow velocity of 0.4 m/s to detach the pre-adhered *L. pneumophila* from biofilms (Figure 4.1). This flow velocity is within the range of real drinking water flow velocity (maximal drinking water flow velocity of 0.7 m/s was obtained by match the design flow rate of 11.4 L/min or 3 GPM of some states in the United States and a common shower pipe size of 0.75 in. or 1.9 cm). Specifically, for the monochloramine treated biofilms, the adhered *L. pneumophila* in biofilms was detached by groundwater containing 0.5 mg Cl₂ mg/L monochloramine. Similarly, groundwater containing 0.5 mg Cl₂ mg/L free chlorine was used to release the *L. pneumophila* from free chlorine treated biofilms. As a control experiment, groundwater free of disinfectant was also used to detach *L. pneumophila* from the monochloramine or free chlorine treated biofilms. For the groundwater biofilms without disinfection treatment, the
pre-adhered *L. pneumophila* were released from biofilms using groundwater, groundwater containing monochloramine, and groundwater containing free chlorine, respectively. For all the above conditions, the groundwater containing or lacking disinfectant was introduced to the flow chamber for 20 minutes. The detached samples (containing detached *L. pneumophila* and sloughed off biofilm materials) were collected at 1, 5, 10, 20 min for further analysis of cultivability and infectivity. The disinfectant in collected samples were immediately quenched using 5% (w/v) sodium thiosulfate.

### 4.3.3 Determination of total released *L. pneumophila* and detached biofilm materials by DNA extraction and qPCR

**Sample preparation and DNA extraction**

For determining the number of detached *L. pneumophila* in the collected samples, DNA of the detached sample collected at each time point was extracted for subsequent qPCR analysis. Specifically, 2 ml of each detached sample was concentrated to 50 µl by centrifuging at 17,000 RCF for two minutes and then carefully removing the supernatant. DNA was then extracted from these concentrated samples using the Qiagen DNA extraction kits (DNeasy Blood & Tissue Kit, Hilden, Germany) following manufacturer’s protocol. After extraction, the DNA was stored in the elution solution provided by the Qiagen DNA extraction kit at -20°C until use.

**Determination of total released *L. pneumophila***

The total concentration of *L. pneumophila* (including all the live cells and disinfection injured cells) in the detached samples were then enumerated by qPCR, following the previous studies.27 The
qPCR reactions were performed in the Applied Biosystems 7900HT Fast Real-Time PCR system. The mixture used in each qPCR reaction (15 µl) contained 2 µl extracted DNA, 200 nM reverse primer, 200 nM forward primer, and 7.5 µl PowerUp™ SYBR® Green Master Mix (Austin, TX). The reverse and forward primers (mip_99F: 5’-GGATAAGTTGTCTTATAGCATTGGTG-3’ and mip_172R: 5’-CCGGATTAACATCTATGCCTTG-3’) were targeted in the macrophage infectivity potentiator (mip) gene of *L. pneumophila*, which were designed in previous study.²⁸ The PCR condition included an initial denaturing step at 95°C for 10 min followed by 40 cycles of 95°C for 10s and 60°C for 1 min as described in previous study. In each qPCR run, 10-fold serial dilutions of DNA standards (5.8×10¹-5.8×10⁷ copy number/ml) were also amplified to create a standard curve. The slope and interception of each standard curve was then used to calculate the copy numbers of target gene in the extracted DNA samples. The possible effect of qPCR inhibitors in the extracted DNA sample was excluded by running qPCR for a series of diluted extracted DNA samples (Figure C1). The qPCR efficiency was ranged from 0.98 to 1.02. The detection limit was from 10¹ to 10⁷ copy numbers. Since a loss of DNA may occur during DNA extraction process, directly using qPCR results (copy numbers) to represent the real concentration of *L. pneumophila* in the detached samples is not accurate. A linear correlation between qPCR results and colony-forming unit (CFU) of *L. pneumophila* was determined based on the qPCR results for non-disinfected *L. pneumophila* samples with known CFU (Figure C2). The final concentration of *L. pneumophila* in each detached sample was reported as C_{Detached} (CFU_qPCR/ml). The details of the method converting between qPCR and *L. pneumophila* CFU was described previously.²⁷

**Determination of total released bacteria**

The number of total released bacteria in the extracted samples were quantified by qPCR following similar procedures described above. The V3 region of the 16S rRNA gene was amplified by
forward primer 341F (5’-CCTACGGGAGGCAGCAG-3’) and reverse primer 518R (5’-ATTACCGCGGCTGCTGG-3’) as described in previous study. The standard curve was generated by serial dilutions (10-fold) of 16S rRNA standards (540bp) through qPCR. To avoid degradation on standards, each set of dilutions of standards was thaw and used immediately after loading all the samples. The dilutions of standards were discarded after each thaw. Dilutions series of standards were prepared from concentrated standards (7.7×10^8 copy number/ml) and molecular grade water and used within one month. The efficiency of the qPCR was in the range of 0.98 to 1.02 in all calibration curves. The detection limit was from 10^1 to 10^7 copy numbers.

4.3.4 Determination of L. pneumophila inactivation

When the biofilm-associated L. pneumophila were exposed to the flowing groundwater containing disinfectant, some released L. pneumophila cells would be injured by the disinfectant and became non-culturables. The amount of culturable L. pneumophila in the detached samples was determined by colony counting on the buffered charcoal yeast extract (BCYE) agar plates with 10 μg/L chloramphenicol. The L. pneumophila cells used in our study carried a chloramphenicol resistance plasmid. Therefore, only the L. pneumophila cells in the detached samples could grow in those chloramphenicol-contained plates, while other detached microorganisms from the biofilms could not grow in those plates. The chloramphenicol concentration of 10 μg/L was selected because this concentration could prevent the growth of bacteria that did not carry the chloramphenicol resistance plasmids, but did not inhibit the growth of L. pneumophila (according to the experiments described in appendix). After each L. pneumophila release experiments, a serial 10-fold dilutions were prepared for each detached sample collected at different time point. 100 μl of each dilution
solution was then dropped and spreaded in a BCYE agar plate. After incubating these plates at 37 °C for 5 days, the number of colonies in each plate was counted. The concentration of culturable \textit{L. pneumophila} in each detached sample (\(C_{\text{Culturable}}\)) was then determined by averaging these colony counting results. In this study, the inactivation of \textit{L. pneumophila} was represented by the ratio of inactivated \textit{L. pneumophila} over concentration of total released \textit{L. pneumophila} as shown below:

\[
\text{Inactivation ratio} = 1 - \frac{C_{\text{Culturable} \text{ L. pneumophila}}}{C_{\text{Total released L. pneumophila}}}
\]  

(1)

where \(C_{\text{Culturable L. pneumophila}}\) (CFU/ml) is the concentration of the culturable \textit{L. pneumophila} in each detached sample determined by colony counting; \(C_{\text{Total released L. pneumophila}}\) (CFU/ml) is the concentration of the total \textit{L. pneumophila} (including culturable and non-culturable \textit{L. pneumophila} cells) in each detached sample determined by qPCR as aforementioned. In this study, the plate counting assay was conducted immediately after the \textit{L. pneumophila} release experiments. Previous study showed that some bacteria cells injured by disinfection may recover after staying in disinfectant-free condition for some time.\cite{29} Therefore, to ensure the plate counting results of released \textit{L. pneumophila} in this study was stable with time, control experiments were conducted to determine the concentration of culturable \textit{L. pneumophila} at 0, 1, and 2 days after disinfection (appendix).

\subsection*{4.3.5 Determination of \textit{L. pneumophila} infectivity}

The infectivity of the detached \textit{L. pneumophila} samples was determined by estimating the ability of \textit{L. pneumophila} reproduction in \textit{Acanthamoeba polyphaga} (\textit{A. polyphaga}, ATCC 30461), an amoeba host of \textit{L. pneumophila} commonly found in natural aquatic environment and water supply.
systems. After each \textit{L. pneumophila} release experiment, 500 µl of the detached sample was added into 4.5 ml liquid culture of \textit{A. polyphaga} culture with a concentration of $8 \times 10^4$ cells/ml. This mixture was then incubated at 30 °C. After three days of incubation, the total amount of \textit{L. pneumophila} in the mixture was determined by DNA extraction and qPCR analysis. The qPCR results were also converted to concentration of \textit{L. pneumophila} (CFU$_{\text{qPCR}}$/ml) using the aforementioned linear model shown in Figure S1. The infectivity of the detached \textit{L. pneumophila} was then calculated by the ratio of concentration of propagated \textit{L. pneumophila} in \textit{A. polyphaga} over the concentration of detached \textit{L. pneumophila}:

\[
\text{Infectivity} = \frac{C_{\text{Total L. pneumophila after infection}}}{C_{\text{Detached}}} \tag{2}
\]

Where $C_{\text{Total L. pneumophila after infection}}$ is the final concentration of the \textit{L. pneumophila} after incubating with \textit{A. polyphaga}.

\subsection*{4.4 Results and discussion}

\subsubsection*{4.4.1 \textit{L. pneumophila} and biofilm materials release kinetics from biofilms}

To estimate the effect of disinfectant exposure on pathogen release by a continuous drinking water flow, the total \textit{L. pneumophila} and other bacteria released from biofilms under disinfectant-free and disinfectant-exposure conditions were compared. Figure 4.2a shows the normalized total \textit{L. pneumophila} released by flowing groundwater, groundwater containing monochloramine, and groundwater containing free chlorine as a function of time. Under all the examined disinfectant/disinfectant-free conditions, the highest \textit{L. pneumophila} release was occurred at the first five minutes of water flow. Subsequently, the release of \textit{L. pneumophila} rapidly reduced in
the first five minutes and then leveled off. For example, by using the disinfectant-free groundwater to wash the biofilms, the release of *L. pneumophila* was decreased from 0.64±0.20 cm⁻¹ to 0.04±0.03 cm⁻¹ in the first five minutes. However, from the fifth to thirtieth minute, the released *L. pneumophila* only changed from 0.04±0.03 cm⁻¹ to 0.03±0.03 cm⁻¹ (t-test, p=0.54). In addition, at most of the time points (1, 2, 5, 10, 15, 20, 30 min), the release of *L. pneumophila* from same groundwater biofilms under disinfectant free, monochloramine, and free chlorine exposure conditions was similar. For example, at the first minute, total *L. pneumophila* released by flowing groundwater, groundwater containing monochloramine, and groundwater containing free chlorine was 0.64±0.20 cm⁻¹, 0.57±0.05 cm⁻¹, and 0.41±0.17 cm⁻¹ (One-way ANOVA, p=0.52). However, at third and fourth minute, the release of *L. pneumophila* under monochloramine exposure (e.g., 0.23±0.03 cm⁻¹ at 4 min) was slightly higher than that under disinfectant-free (e.g., 0.08±0.04 cm⁻¹ at 4 min) or free chlorine (e.g., 0.07±0.05 cm⁻¹ at 4 min) exposure conditions. In summary, the exposure to disinfectant did not significantly altered the release kinetics of *L. pneumophila* from same biofilms.

In addition to determining the total released *L. pneumophila* from biofilms, the total released bacteria cells were also determined to compare the detachment of biofilms materials under disinfectant-free and disinfectant exposure conditions (Figure 4.2b). The change of total released bacteria as a function of time showed similar trend with the change of total released *L. pneumophila*. Specifically, the release of biofilm bacteria decreased dramatically in the first five minutes and then became stable from fifth to thirtieth min. Moreover, while the total released bacteria under disinfectant-free and free chlorine exposure conditions was similar, the total released bacteria under monochloramine exposure condition (red open circle) was higher than that under the other two conditions (blue open triangle and black open square) at the third and fourth
minute. For example, at the third minute, the total released bacteria under monochloramine exposure was \((4.35 \pm 0.76) \times 10^6\) copy number/mL, which is statistically higher than that under disinfectant free \(((1.55 \pm 0.53) \times 10^6\) copy number/mL) and free chlorine exposure \(((1.66 \pm 0.41) \times 10^6)\) copy number/mL) conditions (One-way ANOVA, \(p=0.03\)). The total released \textit{L. pneumophila} and released bacteria as a function of time showed similar trends, suggesting that the release of \textit{L. pneumophila} always accompanied with the release of biofilm materials. Previous studies also suggested that sloughing off biofilm materials can release the pathogens to drinking water.\(^{10, 30}\) In addition, the observation that monochloramine exposure led to higher \textit{L. pneumophila} and biofilm materials release was presumably due to the higher detachment of biofilm cluster occurred at three to four minute. Previous studies showed that monochloramine had better penetration into biofilms thus may lead to higher biomass loss.\(^{31, 32}\) Therefore, compared to disinfectant-free and free chlorine exposure conditions, higher bacteria release under monochloramine exposure was observed at three to four minute.
Figure 4.2 Total a) *L. pneumophila* and b) bacteria released from groundwater biofilms as a function of time. The groundwater (black open square), groundwater containing monochloramine (red open circle), and groundwater containing free chlorine (blue open triangle) were continuously flowing through biofilms and detach *L. pneumophila* and other bacteria from biofilms, respectively. The total released *L. pneumophila* in y axis of a) was calculated by normalizing the concentration of released *L. pneumophila* (#/cm$^3$) by the initially adhered *L. pneumophila* on biofilms (#/cm$^2$). The total released bacteria in y axis of b) was represented by 16S qPCR results (copy number/mL).

In addition to compare the *L. pneumophila* and biofilm materials release under different disinfectant exposure conditions, the release of *L. pneumophila* and biofilm materials from disinfected and non-disinfected biofilms as a function of time was also compared. The long-term
disinfected biofilms used in this study mimicked the biofilms which were exposed to disinfectant-contained fresh drinking water in a distribution or premise plumbing system. On the contrary, the non-disinfected biofilms were used to simulate the drinking water biofilms in some locations where disinfectant residual is lacking or low, such as the stagnant or corroded zones.\textsuperscript{33} As shown in Figure 4.3a, the \textit{L. pneumophila} (normalized by the initial adhered \textit{L. pneumophila}) released from non-disinfected, monochloramine treated, and free chlorine treated biofilms under disinfectant-free condition showed similar trends. For example, at second minute, the total \textit{L. pneumophila} released from non-disinfected, monochloramine treated, and free chlorine treated biofilms was $0.09\pm 0.04 \text{ cm}^{-1}$, $0.05\pm 0.04 \text{ cm}^{-1}$, and $0.003\pm 0.0006 \text{ cm}^{-1}$, respectively (One-way ANOVA, $p=0.19$). The observation that disinfected and non-disinfected biofilms showed similar \textit{L. pneumophila} release trends suggested that pre-disinfecting biofilms did not change the \textit{L. pneumophila} release kinetics. In addition, the release of biofilm materials from non-disinfected, monochloramine treated, and free chlorine treated biofilms was compared in Figure 4.3b. By using the disinfectant-free groundwater to wash the biofilms, biofilm materials released from monochloramine treated and free chlorine treated biofilms was similar. However, higher biofilm materials sloughing off (release) was observed for the non-disinfectant biofilms compared with the monochloramine or free chlorine treated biofilms. Previous study compared the stiffness of long-term disinfected and non-disinfected simulated drinking water biofilms, and found that the biofilms were stiffer after long-term disinfectant exposure.\textsuperscript{22} The stiffer biofilms were expected to detach less because they have better resistance to flow shear stress.\textsuperscript{34} Therefore, under the same flow rate (20 ml/min), the biofilm materials released from monochloramine or free chlorine treated biofilms was lower than those from non-disinfected biofilms.
Figure 4.3 Total a) *L. pneumophila* and b) bacteria released from groundwater biofilms, monochloramine-treated biofilms, and free chlorine-treated biofilms, respectively. The total released *L. pneumophila* in y axis of a) was calculated by normalizing the concentration of released *L. pneumophila* (#/cm²) by the initially adhered *L. pneumophila* on biofilms (#/cm²). The total released bacteria in y axis of b) was represented by 16S qPCR results (copy number/mL).

4.4.2 Inactivation of L. pneumophila released from biofilms

The inactivation of *L. pneumophila* released from biofilms under monochloramine or free chlorine exposure was represented by the ratio of non-culturable *L. pneumophila* to the total released *L.
pneumophila (Figure 4.4). Under either disinfectant exposure, the *L. pneumophila* inactivation as a function of time revealed two regimes. In the first five minutes, *L. pneumophila* inactivation ratio increased rapidly. Specifically, under monochloramine exposure, the ratio of inactivated *L. pneumophila* increased from 0.43±0.11 to 0.90±0.05 in the first five minutes. However, with longer disinfectant exposure, the increase of inactivation ratio leveled off. The ratio of inactivated *L. pneumophila* under monochloramine exposure changed from 0.90±0.05 to 0.95±0.06 during the fifth to thirtieth minute. The observation of these two regimes could be explained by the *L. pneumophila* and biofilm materials detachment process. At the first five minutes, the *L. pneumophila* stayed in the biofilm surface was continuously exposed to disinfectant and detached, thus a rapid increase of the inactivation ratio was observed. However, with longer exposure to the flowing groundwater containing monochloramine, the *L. pneumophila* incorporated to the inner layer biofilms started to be exposed to disinfectant and detached. Those newly exposed *L. pneumophila* cells were not significantly inactivated, thus the inactivation ratio did not dramatically increase from fifth to thirtieth minute.

The inactivation ratio of released *L. pneumophila* from non-disinfected groundwater biofilms under monochloramine and free chlorine was compared (red solid square and blue solid circle in Figure 4.4). The ratio of inactivated *L. pneumophila* released under monochloramine exposure and free chlorine was statistically the same (t-test at each time point, all p>0.05). Previous studies found that monochloramine showed stronger ability of penetrating biofilms than free chlorine, while free chlorine inactivated non-biofilm-associated bacteria more effectively than monochloramine. In this study, either biofilm-associated or non-biofilm-associated *L. pneumophila* was existed and could be inactivated. Therefore, the similar inactivation ratio of *L.
*Pneumophila* released under monochloramine and free chlorine exposure may be caused by the overall effect of biofilm-associated and non-biofilm-associated *L. pneumophila* inactivation.

**Figure 4.4** Inactivation ratio of *L. pneumophila* released from groundwater (GW, non-disinfected) biofilms by monochloramine (red solid square) and free chlorine (blue solid circle). Inactivation ratio of *L. pneumophila* released from monochloramine-treated biofilms by monochloramine (red open square). The inactivation ratio was defined by the ratio of non-cultivable *L. pneumophila* to total released *L. pneumophila*.

In addition to comparing the *L. pneumophila* inactivation under different disinfectant exposure, the ratios of inactivated *L. pneumophila* released from non-disinfected biofilms and monochloramine treated biofilms were compared (red solid square and red open square in Figure 4.4). Overall, the *L. pneumophila* released from monochloramine treated biofilms showed lower inactivation ratios than that from non-disinfected biofilms. For example, at the first minute, the ratios of inactivated *L. pneumophila* released from non-disinfected biofilms was 0.43±0.11, which was statistically lower (t-test, p=0.04) than that from monochloramine-treated biofilms (0.83±0.03). Comparison on the inactivation of *L. pneumophila* released from non-disinfected
biofilms and free-chlorine treated biofilms also showed similar trend (Figure C3). The lower inactivation ratios for *L. pneumophila* released from non-disinfected biofilms suggested that *L. pneumophila* associated with non-disinfected biofilms had better resistance to disinfectant. As shown in the aforementioned results of total biofilm material release (Figure 4.3b), higher sloughing off biofilm materials was observed for the non-disinfected biofilms than the disinfected biofilms. For those *L. pneumophila* released with the sloughing off biofilm materials, the sloughing off biofilm materials could protect *L. pneumophila* from disinfection by limiting the penetration of disinfectant.\(^{35, 36}\) Therefore, higher biofilm materials detachment from non-disinfected biofilms caused the lower ratios of inactivated *L. pneumophila* released from non-disinfected biofilms. Although disinfectant residual was reported to take limited effect on biofilm removal in distribution or premise plumbing systems,\(^{22}\) exposing biofilms to disinfectant could reduce the risk of *L. pneumophila* release when *L. pneumophila* spike occurred.

### 4.4.3 Infectivity of *L. pneumophila* released from biofilms

The infectivity of *L. pneumophila* represented the amplification of *L. pneumophila* population after infecting *A. polyphaga*. The infectivity of *L. pneumophila* released from groundwater biofilms under monochloramine, free chlorine, and non-disinfectant exposure conditions was compared in Figure 4.5a. Under non-disinfectant exposure condition (black square), the *L. pneumophila* infectivity at each time point was statistically the same (One-way ANOVA, p=0.98), suggesting that the infectivity of *L. pneumophila* released from biofilms did not change with time. Specifically, the infectivity was 20±6 at the thirtieth minute, which was constant with the infectivity of 19±5 at the first minute. Compared with the infectivity of released *L. pneumophila* under non-disinfectant
exposure, the *L. pneumophila* infectivity under disinfectant exposure was significantly lower. The infectivity of released *L. pneumophila* under monochloramine and free chlorine conditions was statistically the same (t-test, all p>0.05) and showed similar trend as a function of time. Specifically, under both monochloramine and free chlorine exposure conditions, the infectivity showed a decrease in the first five minutes, indicating that disinfectant exposure gradually decreased the overall *L. pneumophila* population amplification after infecting *A. polyphaga*. The infectivity then became stable from the fifth to thirtieth minute under monochloramine or free chlorine exposure. In addition, at the thirtieth minute of the monochloramine and free chlorine exposure, (4±2) % and (7±2) % of the total *L. pneumophila* cells released from groundwater biofilms were still culturable, respectively (Figure 4.4a). Thus, compared with the *L. pneumophila* released under non-disinfectant condition, the ratio of culturable *L. pneumophila* cells was 25 and 17 times lower under monochloramine and free chlorine exposure, respectively. However, infectivity of *L. pneumophila* released under monochloramine and free chlorine exposure was only 4 and 3 times lower than that under non-disinfectant condition. Therefore, some non-culturable *L. pneumophila* cells also contributed to the *L. pneumophila* population growth after infecting *A. polyphaga*. In other words, part of the non-culturable *L. pneumophila* cells were viable but non-culturable (VBNC) and still had the ability of infecting *A. polyphaga*. The VBNC state was also reported for the planktonic *L. pneumophila* treated with monochloramine, free chlorine, and heat shock.\textsuperscript{37-39} The existence of VBNC *L. pneumophila* released from biofilms under disinfectant exposure suggested that 0.5 mg/L monochloramine and free chlorine exposure may not be sufficient to reduce the infectivity of *L. pneumophila*.

The infectivity of *L. pneumophila* released from non-disinfected groundwater biofilms and disinfected biofilms was also compared, as shown in Figure 4.5b. By using monochloramine-
contained groundwater to release the *L. pneumophila* cells from biofilms, the infectivity of *L. pneumophila* released from groundwater biofilms was significantly higher than that released from the monochloramine treated biofilms. For example, at the end of *L. pneumophila* release experiment, the infectivity of *L. pneumophila* release from groundwater biofilms was 6±0.6, statistically higher than the infectivity of 0.2±0.1 for *L. pneumophila* released from the monochloramine treated biofilms. Comparison on the infectivity of *L. pneumophila* released from non-disinfected biofilms and free-chlorine treated biofilms also showed similar trend (Figure C4). The higher infectivity of *L. pneumophila* released from groundwater biofilms agreed with the lower inactivation ratio of *L. pneumophila* released from same biofilms, further suggested that non-disinfected biofilms can better protect *L. pneumophila* from disinfection.
**Figure 4.5** a) Infectivity of *L. pneumophila* released from groundwater (non-disinfected) biofilms by monochloramine (red open square) and free chlorine (blue open circle). b) Infectivity of *L. pneumophila* released from groundwater biofilms and monochloramine-treated biofilms by monochloramine. The infectivity was defined by the ratio of *L. pneumophila* population after infecting amoeba to *L. pneumophila* population before infecting amoeba.

In summary, this study implied the role of disinfectant exposure on the release, inactivation, and infectivity of biofilm-associated pathogens in drinking water system. Firstly, in real drinking water biofilms, biofilms could developed in both disinfectant exposure and disinfectant-free
conditions. The pathogens could be released from those biofilms and disinfected when the fresh drinking water containing disinfectant flow through the biofilms. Our study indicated that compared with the biofilms developed under disinfectant-free condition, the long-term disinfected biofilms provided less protection for the biofilm-associated pathogens. Although the long-term disinfectant exposure was shown to not effectively remove biofilm biomass,\textsuperscript{13,22} the pre-exposing biofilms to disinfectant could alter the biofilm properties thus reduce the regrowth and infection risk of pathogen released from biofilms. Secondly, the free chlorine and monochloramine exposure did not show obvious difference on inactivating biofilm-associated \textit{L. pneumophila}. While free chlorine is a stronger oxidizer but diffuses less compared to monochloramine,\textsuperscript{31,32} the inactivation efficiency for biofilm-associated pathogens in drinking water system could be attributed to a combined effect of disinfectant oxidation strength and diffusion ability. Finally, the results of this study revealed that part of the inactivated \textit{L. pneumophila} cells can still infect amoeba and propagate with the aid of amoeba. Therefore, even the \textit{L. pneumophila} was inactivated by disinfectant in drinking water, amoeba could increase the risk of \textit{L. pneumophila} reviving. In real drinking water distribution system, \textit{L. pneumophila} can co-exist with amoeba, grow within amoeba, and also be released from amoeba.\textsuperscript{31} In the future study, the interaction between amoeba, \textit{L. pneumophila}, biofilms, and biofilms need to be further investigated. The corresponding risk of \textit{L. pneumophila} co-existing with amoeba and biofilms in drinking water need to be evaluated.

4.5 References


CHAPTER 5: EFFECT OF WATER HARDNESS AND SCALE INHIBITORS ON CHEMICAL COMPOSITION, PHYSICAL STRUCTURE, AND MECHANICAL STIFFNESS OF SIMULATED DRINKING WATER BIOFILMS

5.1 Abstract

Chemical composition, physical structure, and mechanical stiffness of biofilms influence the accumulation and release of pathogens in drinking water distribution system (DWDS). Therefore, understanding the role of drinking water scale control strategies (e.g., hardness reduction and scale inhibitor application) on biofilm properties can provide insights on drinking water pathogen control. In this study, biofilm composition, physical structure, and mechanical stiffness was characterized for the 11 months old biofilms developed from raw groundwater with high hardness, groundwater softened by ion exchange resin, and groundwater containing polyphosphate. The biofilm chemical composition examined by Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA) revealed a higher content of calcium carbonate in groundwater biofilms compared to the biofilms developed from softened groundwater and groundwater containing polyphosphate. This higher calcium carbonate in groundwater biofilms led to the higher biofilm stiffness, which was determined by atomic force microscopy (AFM) and magnetomotive optical coherence elastography (MM-OCE). On the contrary, the biofilms developed from softened groundwater and groundwater containing polyphosphate, which mainly composed by the biopolymers (e.g., protein, lipid, etc.), were found to be softer than the groundwater biofilms. In

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addition, as determined by optical coherence tomography (OCT), the highest biofilm thickness (418 ± 21 µm) was observed for the biofilms developed from groundwater containing polyphosphate, while the lowest biofilm thickness (30 ± 12 µm) was observed for the biofilms developed from softened groundwater. The thick and soft biofilms caused by the application of polyphosphate would be prone to detach and release biofilm-associated pathogens in DWDS. This study showed how biofilm chemical, physical, and mechanical properties vary in response to a complex DWDS environment, which will contribute to strategy development on drinking water microbial safety and DWDS management.

5.2 Introduction

Biofilms in drinking water distribution system (DWDS), composed by extracellular biopolymers, microorganisms, and inorganic particles, can serve as a reservoir of pathogens and raise public health concerns. 1, 2 The pathogens intruded into DWDS can be captured by and embedded in the extracellular polymeric matrix (EPS). EPS excreted by the microorganisms, in biofilm matrix can then protect those pathogens against environmental stress (e.g., disinfectants, hydrodynamics, and low-nutrients).2 The pathogens can then be released from the biofilms during biofilm sloughing off processes. This drinking water pathogen transmission process could be influenced by the biofilm composition, physical structure, and mechanical stiffness. 3-9 For example, the Pseudomonas putida in EPS-rich biofilms showed higher viability than the EPS-lacking biofilms under chlorine exposure.3 Biofilm physical structure, especially biofilm roughness, improved the pathogen accumulation in biofilms. 4-7 The stiffness of biofilms was shown to be essential for detachment of biofilms and biofilm-associated pathogens. 8, 9 In addition, the biofilm composition was reported to take an important role on biofilm structure and mechanical
strength or stiffness. Therefore, comprehensive understanding the biofilm composition, physical structure, and mechanical stiffness as well as the relationship among those biofilm properties can provide insights to strategy development on drinking water microbial safety.

The drinking water components, such as the hardness and scale inhibitors, may affect the biofilm chemical, physical, and mechanical properties. The hardness (Ca\(^{2+}\), Mg\(^{2+}\), etc.) in drinking water can form the crystalized precipitation (e.g., calcium carbonate) to block the pipes and reduce the pipe working life. Therefore, water hardness is controlled to limit the scale formation in DWDS. Previous studies suggested that exposing the membrane reactor biofilms and marine Pseudoalteromonas sp. biofilms to high hardness water could improve the biofilm cohesiveness since calcium can cross-link the biopolymers and bind microorganisms together. However, the chemical composition, structure, and stiffness of biofilms developed under different water hardness conditions was not characterized and compared. In addition to hardness reduction, the scale inhibitors (e.g., polyphosphate) are also added to DWDS to avoid crystallization of calcium carbonate and control scale formation. Meanwhile, the biopolymers (e.g., protein and lipid) in the drinking water biofilms may facilitate or inhibit calcium carbonate crystallization. However, how the scale inhibitor interfere the biofilm composition, structure, and stiffness was not known.

To fill the aforementioned research gaps, this study aimed to investigate how the scale control strategies affect the biofilm composition, structure, and stiffness, and provide guidelines for the improvement of drinking water microbial safety. Two commonly used scale control methods, ion exchange resin treatment and polyphosphate addition, were applied to groundwater and used to obtain the biofilms. In this study, we 1) determined the biofilm chemical composition using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR),
thermogravimetric analysis (TGA), and inductively coupled plasma mass spectrometry (ICP-MS); 2) measured the biofilm structure using optical coherence tomography (OCT); 3) examined the biofilm surface and overall stiffness using atomic force tomography (AFM) and magnetomotive optical coherence elastography (MM-OCE). The results of this study showed how the biofilm chemical, structural, and mechanical properties varied in response to the application of scale control strategies, which would provide insights on DWDS management.

5.3 Materials and methods

5.3.1 Biofilm preparation

Local groundwater, drinking water source of Urbana-Champaign (IL), was used for developing biofilms on PVC surfaces in this study. This groundwater source was treated by greensand filter and mainly contained 1.65±0.08 mM Ca\(^{2+}\), 1.16±0.01 mM Mg\(^{2+}\), and 1.04±0.02 mM Na\(^{+}\). The hardness of the groundwater was 281±8 mg/L. This groundwater had total organic carbon (TOC) of 1-1.6 mg/L and pH of 7.5-7.8. Biofilms used in this study were developed from the raw groundwater with high hardness (hard-groundwater), groundwater with reduced hardness (soft-groundwater), and groundwater containing anti-scalant, respectively. To prepare the groundwater with reduced hardness, 10 L groundwater mixed with 20 g Na\(^{+}\) form ion exchange resin (Amberlite® IR120 Na\(^{+}\) form, Sigma-Aldrich) was stirred overnight. The ion exchange resin was then allowed to settle down under quiescent condition for one hour. The supernatant was then carefully transferred to a new container for subsequent biofilm development. The hardness of ion exchange resin treated groundwater was reduced to 49±1 mg/L (0.16 mM Ca\(^{2+}\) and 0.34 mM Mg\(^{2+}\)). Base on the World Health Organization guidelines for drinking water quality,\(^{16}\) the ion exchange
treated groundwater was considered to be soft water (hardness<60 mg/L). The raw groundwater was considered to be very hard (hardness>180 mg/L). To prepare the groundwater containing scale inhibitor, sodium hexametaphosphate (SHMP, Sigma-Aldrich) was added to groundwater to reach the final concentration of 10 ppm. SHMP is widely used for scale control in drinking water treatment and distribution processes, since SHMP can prevent the crystal growth of calcite precipitation.17 10 ppm of SHMP used in this study was below the maximal use level (11.9 ppm) recommended by National Sanitation Foundation (NSF) (NSF/ANSI 60-2013).

The biofilm developing method was described previously.5,18 Briefly, the hard-groundwater, soft-groundwater, and groundwater containing SHMP was continuously introduced CDC reactors (CBR 90-2, BioSurface Technologies Corporation, Bozeman, MT), respectively. PVC coupons (RD 128-PVC, BioSurface Technologies Corporation, Bozeman, MT) were inserted inside the CDC reactors as substrate to develop biofilms. A shearing condition was maintained during biofilm development process by continuous stirring the CDC reactors at 125 RPM. No extra nutrients or microorganism strains were added into the CDC reactors. Biofilms were developed in CDC reactors for 10 months before further characterization.

5.3.2 Biofilm structure determination using OCT

The thickness and roughness of biofilms developed from hard-groundwater, soft-groundwater, and groundwater containing SHMP was determined by OCT, as described previously.18,19 A custom built 1300 nm based spectral domain OCT system imaged biofilm cross sections with the an axial resolution of 4.2 µm and a transverse resolution of 3.9 µm.20 The images with size of 3.1 by 2.1 mm images were taken with a mode-locked titanium sapphire laser source (Kapteyn-Murnane Laboratories, Inc, Boulder, CO) centered at 800 nm with a bandwidth of 120 nm. For each biofilm
sample, three biofilm coupons were subjected to OCT imaging system immediately after being removed from the reactor. The biofilms were kept in water during the OCT imaging process. Three hundred images were obtained from the three biofilm coupons. Forty five images over the three hundred images for each biofilm sample were randomly selected for further biofilm thickness and roughness analysis. These selected images were processed by Image J (http://imagej.nih.gov/ij/) to eliminate the background. Biofilm mean thickness and roughness was then determined by analyzing grey scale gradient with automatic thresholding using the MATLAB program developed by Derlon et al.21

5.3.3 Biofilm stiffness determination using AFM indentation tests

In this study, biofilm stiffness was represented by Young’s modulus (or elastic modulus). The surface elastic modulus of the biofilms was determined by AFM indentation test, as described previously.22 Briefly, colloidal probe, made by adhering a silica sphere (with diameters of 20 μm) to a tipless cantilever (Calibrated Spring Constant=0.7 N/m, Mikromasch, Lady's Island, SC), was used for indentation tests. All the indentation measurements were performed in sterilized DI water, since the dry biofilms would have different mechanical properties from hydrated biofilms. The contact mode of a MFP-3D AFM (Asylum Research, Santa Barbara, CA) was used for all indentation tests. Before the AFM indentation tests, the biofilm coupons were carefully removed from CDC reactors and then gently rinsed with sterilized DI water for three times. Prior to subjecting the biofilm coupons to indentation tests, the AFM probe was calibrated by measuring force curves on a bare glass surface both in air and in liquid to obtain the cantilever deflection sensitivity for force calculation and to examine the possible contamination.23 After that, the
indentation force was measured in liquid as a function of indentation depth into biofilms. The maximal indentation depth was limited to 3 µm to avoid the interference of PVC substrate.\textsuperscript{24, 25} The indentation curves were then fitted by Hertz model to determine the biofilm elastic modulus.\textsuperscript{23} More details on indentation curve measurement and Hertz model fitting can be found in previous study.\textsuperscript{22}

For each biofilm sample, three biofilm coupons were chosen for indentation tests. On each biofilm coupon, ten locations were randomly selected for elastic modulus determination. On each location, indentation measurements were repeated 10-20 times. Since the biofilms are heterogeneous, the distribution of elastic modulus values obtained from different locations were reported to reveal the surface stiffness of each biofilm sample. The distributions of different biofilm samples were compared statistically using Kolmogorov–Smirnov test.

5.3.4 Biofilm stiffness determination using MM-OCE

MM-OCE method is a novel mechanical property examination tool developed by Professor Boppart research group.\textsuperscript{26, 27} MM-OCE was designed for detecting the unhealthy tissue (e.g. tumors) with different biomechanical properties in-situ. MM-OCE system was composed by an OCT imaging system and a magnetic field generator. In MM-OCE method, magnetic nanoparticles (MNPs) were introduced to the examined materials and served as force transducer in a magnetic field. The motion of examined sample caused by the oscillation of MNPs in the magnetic field could then be monitored by OCT. The oscillation frequency of MNPs could be controlled by adjusting the magnetic field strength. By applying different input MNPs oscillation frequency, the MNPs would induce different oscillation amplitude of examined materials. When the input
frequency was equal to the resonance frequency of examined materials, the examined materials would have maximal oscillation amplitude. This resonance frequency could reflect the stiffness of examined materials and be converted to elastic modulus. Compared to other elastography techniques (e.g., ultrasound elastography and magnetic resonance elastography), MM-OCE has better resolution and requires less detection time.

Unlike AFM determining the biofilm surface stiffness, MM-OCE was also used in this study to measure the overall biofilm stiffness along the biofilm depth. To prepare the MNPs solution, the magnetic iron (II,III) oxide (Fe₃O₄) particles (50-100 nm diameter, Sigma-Aldrich) were dissolved in water to reach the concentration of 2 mg/ml. These MNPs particles in wa(195,573),(768,573),(951,633),(195,633)(195,633),(839,633),(840,691),(195,691)ter phase were aggregated and finally formed the particles with the diameter of ~2 µm. Before the MM-OCE measurement, the examined biofilm coupon were removed from CDC reactors and fixed by a plastic ring mounted in a 50 ml centrifuge tube. To introduce the MNPs into biofilms, 45 ml MNPs solution was added into the centrifuge tube containing the examined biofilm coupon. The centrifuge tube was then slowly rotated at 7 rpm for 10 hours. The biofilm coupon was then carefully taken out of the centrifuge tube, rinsed in DI water for three times, and then immediately subjected to the MM-OCE stage for stiffness measurements. During the MM-OCE measurement, the excitation (input) frequency ranged from 10-500 Hz was applied to the MNPs inside biofilm samples. When the input frequency increased from 10-500 Hz with time, an OCT scan rate of 1983 Hz was chosen to acquire 4000 M-mode scans (the motion of sample with time) at the selected location on biofilm sample. This scan rate allowed a large number of sample scans, and also ensure that the movement of MNPs did not break the sample. The motion (or replacement) of the biofilms at each frequency monitored by the OCT was then converted to the amplitude of biofilms oscillation. A frequency-swept mechanical spectrum was then obtained by plotting the biofilm
oscillation amplitude as a function of input frequency (Figure 5.4). The resonant peak was observed in the frequency-swept curve, showing the resonance frequency of examined biofilms. For each biofilm sample, three biofilm coupons were used for MM-OCE measurement. On each biofilm coupon, 3-4 locations were selected for stiffness determination. In total, MM-OCE data were collected on 9-12 locations for each biofilm sample.

5.3.5 Biofilm composition

A FTIR (PerkinElmer Inc. Waltham, MA) was also used for detect components of biofilms developed from hard-groundwater, soft-groundwater, and groundwater containing SHMP. Before each measurement, the biofilms were scratched from biofilm coupon and dried in air. The dried biofilm materials were then transferred to the FTIR stage and covered the crystal window. During the FTIR measurement, the light absorbance for each biofilm sample was detected over a wide range of spectrum (400-4000 cm\(^{-1}\)). For each biofilm sample, the biofilm preparation and FTIR process was repeated three times, and each FTIR scan was repeated eight to sixteen times. The peaks on the transmittance curve were identified and compared to previous studies.\(^{28-34}\) Surface compositions of the biofilms were then determined due to the position of each peak.

In addition to FTIR, thermogravimetric analysis (TGA, PerkinElmer Inc. Waltham, MA) was conducted to examine biofilm composition. Different biofilm components would pyrolyze and cause the loss of biofilm weight at different temperature. By quantifying the loss weight of biofilms during temperature elevating, TGA can be used to compare the composition of biofilms developed from hard-groundwater, soft-groundwater, and groundwater containing SHMP. Before TGA tests, the biofilms were removed from biofilm coupons and then dried in a 37°C incubator for 4 hours.
The dried biofilms were then transferred to TGA stage and the initial weight of dried biofilms was measured. The weight change over a temperature ranging from 30-800°C at an increase rate of 10°C/min was monitored, and the percentage of remained biofilms (biofilm weight at a certain temperature/initial biofilm weight) was plotted as a function of temperature. All the TGA measurements were conducted in a N2 purge (20 ml/min). Each measurement was duplicated.

Also, inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer – SCIEX ELAN DRCe ICP-MS, Norwalk, CT USA) was used to measure the metallic elements of biofilm samples and reactor feed water. The ICP-MS method was described in SW-846 Test Method 6020B. Before measuring metallic elements of biofilm samples, the biofilms were digested in nitric acid (5% v/v) for two hours. Then the digested biofilm solutions and reactor feed water samples were sent to the Microanalysis Laboratory in University of Illinois at Urbana-Champaign for Ca, Mg, and Na analysis.

5.4 Results and discussion

5.4.1 Biofilm composition

The composition of biofilms developed from hard-groundwater (hard-groundwater biofilms), soft-groundwater (soft-groundwater biofilms), and groundwater containing SHMP (SHMP biofilms) was determined by FTIR and shown in Figure 5.1. From the spectra of hard-groundwater biofilms, three highest peaks were observed at 713 cm⁻¹, 871 cm⁻¹, and 1398 cm⁻¹. The 713 cm⁻¹ peak stood for the calcite or aragonite in biofilms, suggesting that hard groundwater biofilms contained the crystalized calcium carbonate. The 871 cm⁻¹ peak also represented the polymorph of calcium carbonate. According to the previous study, 866 cm⁻¹ represented the out-of-plane carbonate.
(e.g., vaterite), while the 876 cm\(^{-1}\) represented calcite. Thus the peak at 871 cm\(^{-1}\) between 866 and 876 cm\(^{-1}\) was possible to represent the crystal form of calcium carbonate. The peak at 1398 cm\(^{-1}\) could either represent the carbonate or carboxylate ions.\(^{33,36}\) In addition these three high peaks, small peaks at 1084 cm\(^{-1}\) and 1646 cm\(^{-1}\) were also found. These two peaks represented the ring variations of polysaccharide (1084 cm\(^{-1}\)) and unordered protein (1646 cm\(^{-1}\)), respectively.\(^{28,33}\) Polysaccharide and protein are two of the main biofilm components. However, the polysaccharide and protein peaks in hard-groundwater biofilms were not significant, probably due to high content of calcium carbonate species.

Compared with the hard-groundwater biofilms FTIR spectra, the spectra of soft-groundwater biofilms did not show high peaks representing calcium carbonate species, but revealed high content of organic components. Two small peaks at 873 cm\(^{-1}\) and 1409 cm\(^{-1}\) suggested that the soft-groundwater biofilms may contain some calcite and ACC. In this spectra for soft-groundwater biofilms, the highest peak was found at 1636 cm\(^{-1}\), representing the Amide I group in some proteins (e.g., carbonic anhydrase and trypsinogen).\(^{28}\) The peak at 1084 cm\(^{-1}\) representing polysaccharide was also found for the soft-groundwater biofilms. Besides protein and polysaccharide, two peaks at 2852 cm\(^{-1}\) and 2922 cm\(^{-1}\) were observed, representing the fatty acid in biofilms.\(^{33}\) Similarly, in the spectra of SHMP biofilms, the peaks representing protein and fatty acid were also observed. An obvious peak observed at 1403 cm\(^{-1}\) was observed, suggesting the SHMP biofilms may contain some ACC.\(^{31}\) In addition, two high peaks were observed at 1030 cm\(^{-1}\) and 1235 cm\(^{-1}\). The 1030 cm\(^{-1}\) and 1235 cm\(^{-1}\) peaks in FTIR spectra were also reported previously for the pure sodium tripolyphosphate and SHMP solutions.\(^{29}\) Thus, the observation of 1030 cm\(^{-1}\) and 1235 cm\(^{-1}\) peaks revealed the polyphosphate species in SHMP biofilms. In summary, the FTIR analysis revealed the existence of crystalized calcium carbonate in hard-groundwater biofilms, while the soft-
groundwater and SHMP was found to contain more organic components. However, FTIR analysis can only be used to qualitatively determine the components in biofilms instead of quantifying the biofilm components.

![FTIR spectra of hard-groundwater biofilms, soft-groundwater biofilms, and SHMP biofilms.](image)

**Figure 5.1** FTIR spectra of hard-groundwater biofilms, soft-groundwater biofilms, and SHMP biofilms. To further compare the biofilm composition quantitatively, the biofilm weight change during pyrolysis

To quantitatively determine the amount of biopolymers (e.g., protein, polysaccharide and lipid) and inorganic components (e.g., CaCO₃) in biofilms, the biofilm weight change as a function of increasing temperature (TGA curve) was plotted (Figure 5.2a). For the TGA curve of soft-groundwater and SHMP biofilms, most of the biofilm weight loss occurred at the temperature...
range of 250-560 °C; while the most weight loss of the hard-groundwater biofilms happened at the temperature range of 620-810°C. To show the stages of biofilm decomposition process more clearly, the derivative biofilm weight (showing the rate of biofilm weight loss) was plotted as a function of temperature (DTG curve, Figure 5.2b). The soft-groundwater and SHMP biofilms showed similar DTG curves, on which two peaks were observed. For example, for the soft-groundwater biofilms, the first peak across the temperature range of 37-173 °C revealed a 9.6% of biofilm weight loss. In this temperature range, the retained water after pre-drying biofilms as well as some highly volatile compounds was eliminated. The second peak throughout the temperature range of 232-559 °C corresponded to a weight loss of 43.8 %. The loss of biomass organic compounds, including microalgae, protein, and lipids, were reported to mainly happen at the temperature range of 200-600 °C. The biofilm biomass, which has similar types of organic compounds (e.g., protein, carbohydrate and lipid), was expected to be decomposed at similar range. Therefore, the second peak on the DTG curve revealed the loss of biopolymers. Unlike the soft-groundwater and SHMP biofilm DTG curves, the hard-groundwater biofilm DTG curve exhibited three peaks. The first peak (36-102 °C) corresponded to 0.6% weight loss of water and highly-volatile compounds. The second peak (241-574 °C) revealed the 5.5% weight loss of biopolymers. The third peak (624-877 °C) corresponded to 40.8% weight loss, which caused by the inorganic compounds. The FTIR revealed that hard-groundwater biofilms contained CaCO₃. Also, the ICP-MS analysis showed higher Ca element content in hard-groundwater biofilms (0.04 µg/mm³) than that in soft-groundwater biofilms (0.0007 µg/mm³) and SHMP biofilms (0.02 µg/mm³). Therefore, the third peak in hard-groundwater DTG curve suggested the high content of CaCO₃, which was not observed in soft-groundwater or SHMP biofilms.
Figure 5.2 a) TGA curve showing the percentage of remained biofilms (biofilm weight at a certain temperature/initial biofilm weight) as a function of temperature and b) derivative thermogravimetric analysis (DTG) curve showing the change of biofilm decomposition rate as a function of temperature.

In summary, FTIR combined with TGA analysis suggested high CaCO₃ contents in hard-groundwater biofilms, while the soft-groundwater and SHMP biofilms were found to mainly contain organic components (e.g., biopolymers). For the hard-groundwater biofilms developed from water source containing high concentration of Ca²⁺, the high CaCO₃ contents was due to the precipitation of Ca²⁺. Previous studies reported that the CaCO₃ precipitation can be induced by bacteria or biofilms.⁴¹-⁴³ These CaCO₃ precipitates can accumulate inside the biofilms and serve as the support for biofilms development.⁴⁰ Therefore, the hard-groundwater biofilms contained the
crystal form of CaCO$_3$. However, for the biofilms developed from softened groundwater containing low level of Ca$^{2+}$, the calcium precipitation and accumulation was reduced thus the soft-groundwater biofilms contained little inorganic precipitates. For biofilms developed from groundwater containing SHMP, the SHMP prevented precipitation of CaCO$_3$. Therefore, although considerate amount of Ca element was observed in SHMP biofilms (0.02 µg/mm$^3$), no calcite peak was observed in the FTIR spectra. Instead, the Ca may be existed in biofilms in the form of organic calcium or ACC.

5.4.2 Biofilm structure

The average thickness and relative roughness coefficient of the biofilms developed from hard-groundwater, soft-groundwater, and groundwater containing SHMP (Figure 5.3). Compared to the thickness of biofilms developed from hard and soft-groundwater, the biofilms developed from groundwater containing SHMP (SHMP biofilms) were much thicker. Specifically, the thickness of SHMP biofilms (418±21 µm) was 6 times higher than the hard-groundwater biofilms and 14 times higher than the soft-groundwater biofilms. The observation that SHMP biofilms showed highest thickness was because polyphosphate could support bacteria growth, contribute to bacteria metabolic regulation, and help bacteria to resist environmental stress.$^{15, 44-46}$ Comparison to the biofilms developed from hard-groundwater, biofilms developed from soft-groundwater were thinner (72±10 µm vs. 30±12 µm, t-test, $p=9\times10^{-21}$), probably due to the loss of organic matter in groundwater during the ion exchange resin treatment process. In addition to biofilm thickness, the roughness of the hard-groundwater biofilms, soft-groundwater biofilms, and SHMP biofilms was also compared. The highest relative roughness coefficient of 0.71±0.15 was observed for the soft-groundwater biofilms. The SHMP biofilms showed the lowest roughness coefficient of 0.19±0.04,
suggesting that adding SHMP to groundwater led to smooth biofilm surfaces. In summary, SHMP biofilms were the thickest and smoothest among all the examined biofilms.

**Figure 5.3** a) Average thickness, relative roughness coefficient and b) selected OCT images of biofilms developed from hard-groundwater, soft-groundwater, and groundwater containing SHMP.
5.4.3 Biofilm stiffness

The surface stiffness, which was represented by elastic modulus frequency distribution, was determined for hard-groundwater biofilms and soft-groundwater biofilms by the AFM indentation tests (Figure 5.4a and b). Compared to the elastic modulus of hard-groundwater biofilms, the elastic modulus of soft-groundwater biofilms concentrated concentration in a lower value range (Kolmogorov-Smirnov test, $p=8\times10^{-12}$). Specifically, for the hard-groundwater biofilms, the maximal elastic modulus frequency located in the range of 3-4 kPa, while the elastic modulus values of soft-groundwater biofilms mainly distributed in the range of 0-1 kPa. Elastic modulus distributing in higher range indicated that the biofilms developed from high hardness groundwater was stiffer than those developed from softened groundwater. The AFM indentation tests clearly showed the difference in stiffness of biofilms developed from hard-groundwater and soft-groundwater. However, using AFM indentation tests on biofilm stiffness determination has some limitations: 1) only the biofilm surface layer stiffness, instead of the overall biofilm stiffness, can be quantitatively determined; 2) it is challenging to probe the very soft and highly hydrated samples in liquid phase. In this study, AFM indentation tests failed to measure the surface stiffness for the SHMP biofilms, since the SHMP biofilms were fluffy and highly flexible in water phase. Therefore, to compare the overall stiffness of SHMP biofilms with the other two biofilms, the MM-OCE method was further conducted and discussed.
The frequency distributions of elastic modulus values for a) the hard-groundwater biofilms and b) soft-groundwater biofilms

The biofilm overall stiffness could be reflected by the biofilm resonance frequency measured by MM-OCE. The material oscillation amplitude over the wide frequency range from 10 to 500 Hz was plotted for the hard-groundwater, soft-groundwater, and SHMP biofilms in Figure 5.5a. The peak in each amplitude curve revealed the frequency which caused maximal biofilm oscillation, which was the biofilm resonance frequency. For the hard-groundwater biofilms amplitude curve shown in Figure 5.5a, the peak was observed at 287 Hz. By analyzing 28 amplitude curves for hard-groundwater biofilms, the average resonance frequency of the hard-groundwater biofilms was 287 Hz (Figure 5.5b). Compared to the hard-groundwater biofilms, the soft-groundwater and SHMP showed lower resonance frequency. Specifically, the average frequency of 200 Hz and 178 Hz was observed for the soft-groundwater and SHMP biofilms,
respectively. The lower resonance frequency of soft-groundwater and SHMP biofilms suggested that these two biofilms were less stiff than the hard-groundwater biofilms.

![Figure 5.5](image)

**Figure 5.5** a) The selected biofilm oscillation amplitude curves at the frequency ranged from 10 to 500 Hz. The peak in the curve showed the resonance frequency of each biofilms. b) The average resonance frequency of each biofilm samples.

AFM indentation testes revealed that the surfaces of hard-groundwater biofilms were stiffer than the soft-groundwater biofilms, while MM-OCE measurements showed that the overall stiffness of the hard-groundwater biofilms were the highest among all the examined biofilms. The high stiffness of the hard-groundwater biofilms may be due to the high content of CaCO₃. The FTIR analysis revealed the existence of crystalized CaCO₃, such as calcite and aragonite, in the hard-groundwater biofilms. These crystalized CaCO₃, which had high stiffness, contributed to the stiffness of hard-groundwater biofilms. Therefore, the overall hard-groundwater biofilms were stiffer than the soft-groundwater and SHMP biofilms. In addition, the higher surface stiffness of
hard-groundwater biofilms than soft-groundwater biofilms may also be attributed to the high concentration of Ca\(^{2+}\) in non-treated groundwater. The Ca\(^{2+}\) in the liquid phase was suggested to bind the biopolymers and improve the cohesiveness of biofilms.\(^{11, 47}\) Therefore, the soft-groundwater biofilms developed from low Ca\(^{2+}\) environment had lower surface stiffness than hard-groundwater biofilms.

In summary, this study revealed that the biofilms developed from high-hardness water source would have high content of CaCO\(_3\). The CaCO\(_3\) took an important role on improving biofilm stiffness. The stiff biofilms would be expected to have less detachment under drinking water flow, thus lead to less biofilm-associated pathogen release. However, the CaCO\(_3\) precipitation induced by and accumulated in the biofilms may also block the drinking water flow in pipes. If the hardness of water source was reduced, less biofilms and little CaCO\(_3\) precipitation would be accumulated on the pipe surfaces thus low pipe blocking would be expected. In addition, adding polyphosphate to water source can also reduce the CaCO\(_3\) accumulations. However, the polyphosphate in water source led to the high volume and low strength of biofilms, which may improve the risk of biofilm detachment and microbial contamination in drinking water.

In this study, we mainly discussed the relationship between the biofilm chemical composition and mechanical stiffness. In the next step, the effect of biofilm microbial community and spatial distribution on biofilm stiffness need to be investigated. Specifically, different bacteria species in biofilm could secrete different organic substances to build biofilm matrix. Therefore, the biofilm composition and mechanical strength may also be determined by the biofilm microbial community. Understanding how the drinking water source chemistries (e.g., hardness, pH, TOC) alter the biofilm microbial community\(^{48}\) and thus affect the biofilm stiffness is needed for drinking water safety control. In addition, the biofilm bacteria spatial distribution (e.g., bacteria density) could
decide the biofilm mechanical cohesiveness.\textsuperscript{49} The knowledge on whether the biofilms developed from different drinking water sources may have different bacteria spatial distribution and thus influence biofilm mechanical stiffness is also necessary for drinking water management.

5.5 References

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CHAPTER 6: SUMMARY AND CONCLUSIONS

*L. pneumophila* can transmit and persist in drinking water distribution system (DWDS) with the aid of drinking water biofilms. Once *L. pneumophila* cells adhere onto biofilms, biofilms can provide a favorable environment for *L. pneumophila* by supplying nutrients and protecting *L. pneumophila* from disinfection. Subsequently, the sloughing biofilms and flowing drinking water can release the biofilm-associated *L. pneumophila* cells to consumers. Therefore, comprehensive understanding of the factors affecting *L. pneumophila* transmission is necessary for *L. pneumophila* control in DWDS.

In this research, we aimed to elucidate the mechanisms controlling the accumulation, disinfection, and release of *L. pneumophila* associated with biofilms under the simulated DWDS conditions. We investigated how the biofilm properties affect the transmission of *L. pneumophila* and identified that biofilm structure as well as biofilm stiffness can influence the accumulation, disinfection, and release of biofilm-associated *L. pneumophila*. Also, we further investigated how the factors from drinking water environment (e.g., chemistries and hydrodynamics) affect biofilm properties. This comprehensive research considered the interaction between *L. pneumophila*, biofilms, and drinking water, which provided information for pathogen control and drinking water management.

6.1 Conclusions

First, the biofilm roughness was proved to enhance the adhesion of *L. pneumophila* to biofilms and prevent the release of *L. pneumophila* from biofilms. The adhesion of *L. pneumophila* was
observed to positively correlate with biofilm roughness due to enlarged biofilm surface area and local flow conditions created by roughness asperities. The release of *L. pneumophila* from smoother biofilms was higher than that from rougher biofilms, presumably because of the low shear stress zones near roughness asperities. Biofilm physical structure was identified as a key factor influencing *L. pneumophila* adhesion to and release from simulated drinking water biofilm.

Second, long-term disinfectant exposure could not reduce biofilm thickness. However, the biofilms were stiffened after long-term disinfectant exposure. Within a relative short term disinfectant exposure (1 month), biofilms were stiffened and thinned. However, the biofilms became softer and thicker again with longer disinfectant exposure (2-3 months), suggesting that biofilms microbial communities can adapt to disinfectant exposure. Overall, the biofilm thickness before and after 3 months of disinfection was similar, while the biofilm stiffness after 3 months disinfection was higher than that before disinfection. These stiffened biofilms after disinfectant would have better resistance to flow shear stress, thus less biofilm detachment and less biofilm-associated pathogen release would be expected.

Third, compared to the pre-disinfected biofilms, the non-disinfected biofilms can provide better protection for *L. pneumophila* from disinfection. In DWDS, biofilms are possible to be continuously exposed to disinfectant (pre-disinfected biofilms) or rarely exposed to disinfectant (non-disinfectant biofilms). When the fresh drinking water containing disinfectant flow through those biofilms, the *L. pneumophila* associated with biofilms could be released and disinfected. The inactivation of *L. pneumophila* released from pre-disinfected biofilms was higher than that from non-disinfected biofilms; while the infectivity of *L. pneumophila* released from pre-disinfected biofilms was lower than non-disinfected biofilms. Non-disinfected biofilms can provide better protection for *L. pneumophila* under disinfectant exposure, probably because more biofilm
materials detached from the softer non-disinfected biofilms, surrounded the released *L. pneumophila*, and separated the *L. pneumophila* from disinfectant.

Finally, reducing water hardness and applying scale inhibitor to water diminished the calcium carbonate precipitating inside biofilms, thus led to biofilms with low stiffness. On the contrary, biofilms developed from high hardness groundwater had the high content of calcium carbonate precipitation, thus showed the high stiffness. While the scale inhibitor (polyphosphate) can facilitate the growth of microorganisms, thick biofilms were developed from groundwater containing scale inhibitor. These thick and soft biofilms would be expected to release more microorganisms in DWDS, thus cause drinking water microbial safety concern.

### 6.2 Contributions

Pathogens in DWDS make huge threat to drinking water safety. However, the factors controlling pathogen transmission in DWDS is not well recognized. Comprehensively understanding of pathogen persistence and transport in DWDS is urgently needed for the development of drinking water safety strategies. To fill the research gaps, this research investigated the transmission of *L. pneumophila*, the pathogen which made biggest contribution to drinking water disease outbreaks in US, under simulated DWDS conditions. The relation between *L. pneumophila*, biofilms, and drinking water was discussed, which could improve the current knowledge on *L. pneumophila* transmission. For example, the disinfectant exposure was found to have limited effect on removing biofilms. However, the biofilms became stiffer after disinfection and thus release less biofilms and also provide less protection to pathogens. The improved understanding on the transmission of biofilm-associated *L. pneumophila* under various drinking
water conditions would provide insights for the drinking water management and development of
pathogen control.

The results of this research were obtained from representative DWDS conditions and thus
could provide valuable information for the \textit{L. pneumophila} risk assessment in drinking water. The
flow hydrodynamic and disinfectant exposure conditions in real DWDS were mimicked in the \textit{L. pneumophila} adhesion, disinfection, and release experiments. Also, the all the biofilms used in this
study were developed from low-nutrient groundwater, the local drinking water source in
Champaign-Urbana, which could well represent the multi-species drinking water biofilms in
practice. The results and conclusions obtained from this research contribute to the evaluation of \textit{L. pneumophila} release and exposure level from drinking water system, thus facilitate the risk
assessment and risk communication.

Finally, the tools and techniques used in this study are transferrable to the other related
environmental engineering field. Specifically, this research applied AFM micro-indentation to
measure biofilm surface stiffness, providing a promising and non-destructive way to determine
micro scale drinking water biofilm mechanical properties in a liquid environment. In addition, the
MM-OCE method, which detected biofilms overall stiffness with the aid of external magnetic field,
was applied on biofilm overall stiffness for the first time. The MM-OCE method enables the
potential non-destructive analysis for real DWDS biofilm stiffness in situ.

\textbf{6.3 Limitations and future prospects}

This research identified the effect of drinking water components on biofilm physical properties,
including biofilm structure and stiffness. However, how the biofilm microbial community
responds to drinking water chemistries was not investigated in this study. The biofilm microbial community change during the disinfectant exposure can help to elucidate the mechanisms of how biofilms establish the resistance to disinfection. In addition, the water hardness and scale inhibitor may also influence the microbial community. Identifying the microbial community under different water chemistry conditions can provide insights on drinking water microbial safety thus facilitate drinking water management. Therefore, the future work should explore the possible microbial community change corresponded to the drinking water components.

The infectivity of \textit{L. pneumophila} released from biofilms was evaluated in this study by amoeba infection assay. Actually, amoeba can also attach to biofilms and co-existed with \textit{L. pneumophila}, thus lead to high risk of \textit{L. pneumophila} propagation in DWDS. However, the interaction between amoeba, \textit{L. pneumophila}, and biofilms was not investigated in this research. The mechanisms controlling \textit{L. pneumophila} infecting amoeba and replicating in DWDS was not identified yet. Thus the future work should explore the transmission of \textit{L. pneumophila} in DWDS in presence of amoeba.

The results of this research provided quantitative information on the accumulation, release, and disinfection of \textit{L. pneumophila} associated with biofilms in drinking water distribution system. Future work can utilize these \textit{L. pneumophila} transmission information on quantitative microbial risk assessment (QMRA) for \textit{L. pneumophila} in drinking water. By assessing the health impact, QMRA could facilitate the risk communication between academia, government, water industry, and public, thus improve the development of drinking water safety strategy.

Also, this research focused on the fate and transport of \textit{L. pneumophila} after \textit{L. pneumophila} entering the water distribution or premise plumbing systems. For eliminating the disease outbreaks caused by \textit{L. pneumophila} in drinking water, how \textit{L. pneumophila} intrudes into DWDS or premise
plumbing systems need to be further investigated. Also, for detecting the *L. pneumophila* prior to the disease outbreaks, the fast portable *L. pneumophila* detection tools used to monitor *L. pneumophila* in DWDS are also need to be developed. In addition, the nutrient sources in DWDS for *L. pneumophila* growing need to be identified for *L. pneumophila* control.

At last, *L. pneumophila* infects human body through the aerosolization of drinking water containing *L. pneumophila*. The factors controlling the pathogen aerosolization need to be identified. Also, the emergent *L. pneumophila* control strategies, considering the transmission in both drinking water and air, need to be developed.
APPENDIX A: SUPPORTING INFORMATION FOR CHAPTER 2

Biofilm preparation

CDC reactor is a continuous flow stirred-tank reactor developed by Centers for Disease Control and Prevention (CDC),\(^1\) and was frequently used for growing biofilms.\(^2\)\(^-\)\(^5\) The CDC biofilm reactors were fed continuously with groundwater at a flow rate of 1.30 mL/min, providing a hydraulic retention time of about 4.5 hours, as recommended by American Society for Testing and Materials (ASTM) standard.\(^6\) The CDC reactor contents were continuously mixed by a magnetic stirrer bar operating at 125 rpm to provide shear on the surface of the PVC coupons. Biofilms were grown for 2, 4, 8, 14, 29, 30, and 34 weeks, and the coupons were removed from the CDC reactors for subsequent experiments. The 2 to 29-week biofilms were harvested in Spring 2013 and used in adhesion experiments, while the 30 and 34-week biofilms were harvested in Spring 2014 used in detachment experiments. Based on our previous findings using the same reactors and groundwater source,\(^7\) after eight weeks, biofilms completely covered PVC surfaces, and microbial communities and thickness became stable.

\(L.\ pneumophila\) cell characterization

\(L.\ pneumophila\) cells were cultured in buffered yeast extract (BYE) medium with a supplement of 10 \(\mu\)g/mL chloramphenicol. Cells were harvested after a 48-hour incubation at 37 °C, and washed by centrifuging at 17000×g and re-suspending in sterile deionized (DI) water three times. The cells were then re-suspended in potassium chloride (KCl) at different ionic strengths (3-300 mM) and buffered with 1 mM NaHCO\(_3\) (pH 8.2-8.4) for subsequent adhesion experiments. The membrane permeability of \(L.\ pneumophila\) cells and their electrophoretic mobility, which can influence cell adhesion, were also characterized. The membrane permeability of \(L.\ pneumophila\) cells was
determined by green and red fluorescent nucleic acid stains (SYTO® 9 and propidium iodide) supplied with LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Grand Island, NY, USA). SYTO® 9 stains all cells in population, while PI only labels cells with damaged membranes.\textsuperscript{8,9} Before staining, equal amounts of SYTO® 9 and PI were mixed together and diluted 10 times to make the stain stock solution. The stock solution was always protected from light. For dying \textit{L. pneumophila} cells, the cells were firstly suspended in 100 mM KCl solution with a concentration of 10\textsuperscript{5} cells/mL. 5 µL stain stock solution was then added into 500 µL of \textit{L. pneumophila} cells solution. After incubation in the dark for 15 min, the unbound stain was washed by adding 10 mL 100 mM KCl solution, and the stained cells were then retained on a black polycarbonate membrane (0.2 µ pore size) by filtration. The membrane was then fixed on a glass slide with BacLight\textsuperscript{™} mounting oil (Invitrogen, Eugene, OR, USA). The Zeiss fluorescence microscope was used to image the stained cells, and the ratio of live/total cells was determined by counting the green and red cells in these images. Electrophoretic mobility of \textit{L. pneumophila} in buffered KCl solutions at different ionic strengths ranging from 3 to 300 mM was determined by a Zetasizer Nano ZS90 (Malvern Instruments, UK). For measuring electrophoretic mobility of \textit{L. pneumophila}, \textit{L. pneumophila} cells were suspended in the desired electrolyte solutions at a concentration of 1-5×10\textsuperscript{7} cells/mL.

\textbf{Adhesion experiment and Sherwood number calculation}

Adhesion experiments of GPF-tagged \textit{L. pneumophila} cells on 2, 4, 8, 14 and 29-week biofilms and PVC surfaces were conducted using a parallel plate flow chamber (FC 71, BioSurface Technologies Corporation, Bozeman, MT). The distance between biofilm surface and the glass cover slide was determined by OCT imaging to be around 0.2 mm. The width of flow chamber is
13 mm, and the length is 30 mm. The imaging area of 0.395×0.296 mm in the center of each biofilm coupon was chosen in adhesion experiments.

Before each experiment, the flow chamber containing coupons with or without biofilm was fixed on the stage of an inverted microscope, and then pre-equilibrated with the desired electrolyte solution. After pre-equilibrating for 2 min, the corresponding electrolyte solution with 1-5×10^7 cells/mL of \textit{L. pneumophila} was pumped into the flow chamber at an average flow velocity of 0.007 m/s for 30 minutes. The number of \textit{L. pneumophila} cells adhering to biofilms was determined with the aid of an epi-fluorescence microscope or a confocal laser-scanning microscope (CLSM). For experiments using the fluorescence microscope (Leica DM15000 M), the images of biofilm surface with adhered cells were taken at 1 min intervals throughout the 30 minutes’ adhesion experiments. Focus was adjusted manually throughout the experiments to capture the biofilm surfaces. Adhered cells were counted from all images to determine adhered cells with time. For experiments with CLSM (TCS SP2 RBB, Leica Microsystems), real-time determination of adhered cells was not possible because this method requires time to scan the biofilm at different depths. Instead, the 3-dimensional image of adhered cells through the whole biofilm body was obtained. Adhesion experiments on selected (2-, 14-, and 18-week) biofilms were each conducted with 3, 10 and 100 mM KCL electrolyte solutions. Immediately after the 30-min adhesion experiment, the same electrolyte solution was pumped into the flow cell to remove cells that did not adhere to the biofilm surface. After 2 min of rinsing, the biofilms were carefully transferred from the flow cells to the CLSM setup for imaging. The 2-dimensional CLSM horizontal section images were reconstructed into a 3-dimensional image with the software of Imaris, and the number of total adhered \textit{L. pneumophila} cells was counted. On each PVC coupon grown with biofilms, 6-9 locations were picked for CLSM imaging. For each combination of
biofilm age and ionic strength, adhesion experiments were repeated three times. Examples of fluorescent microscope and CLSM image for the adhered *L. pneumophila* cells on biofilms were shown in Figure S1.

The adhesion kinetics was expressed as Sherwood numbers, which represent the average local particle transfer rate to the collector surface.\textsuperscript{10-13} The Sherwood number was calculated as the ratio of experimentally determined cell adhesion mass transfer divided by diffusive mass transfer of the cells, and used to present adhesion data so that the dataset obtained could be compared with previous work.\textsuperscript{10-15}

\[
Sh = \frac{J \times a}{C_0 \times D}
\]  

(1)

where \(J\) is the flux of *L. pneumophila* cells (#/m\(^2\) s) calculated as cell adhesion rate per unit viewing area, \(a\) is the equivalent radius of *L. pneumophila* cell (m) (\(a=0.83\ \mu\text{m}\), calculated from the average cell width of 1\(\mu\text{m}\), cell length of 3.1\(\mu\text{m}\), and the radius of the round end of 0.36 \(\mu\text{m}\)), \(C_0\) is the initial *L. pneumophila* cell concentration (#/m\(^3\)), and \(D\) is the diffusion coefficient of *L. pneumophila* cells \((2.92 \times 10^{-13}\text{m}^2/\text{s})\) calculated from Stokes-Einstein equation.\textsuperscript{16} Because Sherwood number is the ratio of mass transfer of cell adhesion to the cell diffusion mass transfer, \(Sh=1\) means mass transfer of cell adhesion onto biofilm surface is equal to cell diffusion mass transfer.

**Re number calculation for flow chamber**

Adhesion and detachment of *L. pneumophila* cells on biofilms and PVC surfaces were examined in a coupon evaluation flow chamber (Biosurface Technologies Corp. FC 71). Reynolds numbers
(Re) for flow chamber at all of the flow conditions used in adhesion and detachment experiments were calculated using the following equations\textsuperscript{16, 17}:

\[
Re_{\text{flow chamber}} = \frac{Q \times \rho}{(w + 2b) \times \mu}
\]

(2)

Where Q is the flow rate (m\textsuperscript{3}/s), \(\rho\) is the flow density (kg/m\textsuperscript{3}), \(\mu\) is the viscosity of the electrolyte solution used in the experiments (kg\cdot m\textsuperscript{-1}\cdot s\textsuperscript{-1}), w is the width of flow chamber (m), and b is the half depth between coupon surface and glass cover surface (m). In this study, w is 0.013m and b is 0.0001m. The calculated Re\textsubscript{flow chamber} are 1.26 at flow velocity of 0.007 m/s, 18.9 at flow velocity of 0.1 m/s, 50.4 at flow velocity of 0.3 m/s and 126 at flow velocity of 0.7 m/s. These Re numbers indicated that all the adhesion and detachment experiments were conducted at Laminar flow condition.

**Biofilm average thickness and relative roughness coefficient calculation using OCT imaging**

Biofilm mean thickness and relative roughness coefficient were obtained by analyzing 20-25 OCT images for a given biofilm with the program developed by Derlon et al.\textsuperscript{18} This program distinguished the PVC-biofilm interface, binarized the image by grey-scale gradient analysis and automatic thresholding, and gave the values of biofilm thickness (\(z_i\) in \(\mu\)m). Then the mean biofilm thickness (\(\bar{z}\) in \(\mu\)m), relative roughness (\(R_{a'}\) ) coefficients, and surface enlargement were calculated according to the following equations.

\[
z = \frac{1}{n} \sum_{i=1}^{N} z_i
\]

(3)
Biofilm surface enlargement coefficient, a parameter representing biofilm surface area, was defined as the ratio of biofilm surface area and the substratum PVC area. The surface enlargement coefficients for the roughest biofilms (4-week) and the smoothest biofilms used in adhesion experiments were calculated from dividing the length of the biofilm contours by the length of corresponding PVC substratum.

The final thickness was corrected with the refractive index of the biofilm, determined by using the method described by Zysk et al. For five locations in 8-week old groundwater-grown biofilms, the reflective index was experimentally determined to be 1.4±0.2.

**L. pneumophila detachment quantification**

During the detachment experiments for *L. pneumophila*, the number of retained cells \(N_t\) on biofilm surfaces at each imaging time point was counted. The ratio of retained cells \(R_t\), which was calculated by dividing the number of retained cells \(N_t\) with the number of initial adhered cells \(N_0\), was presented as a function of time. The final retained cell ratio \(R_{final}\), final detached cell ratio \(D_{final}\), and the time for 90% of maximal cell detachment \(T_{90}\) was determined according to the equations below:

\[
R_t = \frac{N_t}{N_0} \quad (5)
\]

\[
R_{final} = \frac{N_{30min}}{N_0} \quad (6)
\]
\[ D_{final} = 1 - R_{final} \] (7)

\[ R_{90} = 1 - 90\% \times (1 - R_{final}) \] (8)

\[ T_{90} = T_{R_t=R_{90}} \] (9)

where \( N_{30\text{min}} \) is the number of retained cells at the time of 30 min, and \( R_{90} \) is the retained cell ratio when 90% of total detached cells left the biofilm surface.

**Diffusion coefficient of L. pneumophila**

The diffusion coefficient of \( L. \) pneumophila cells (m\(^2\)/s) calculated from Stokes-Einstein equation.\(^{16} \)

\[ D = \frac{K \times T}{6\pi \times \mu \times a} \] (10)

Where \( K \) is Boltzmann constant (J/K), \( T \) is temperature used in the CLSM deposition experiments (K), \( \mu \) is the viscosity of the electrolyte solution used in the experiments (kg\( \cdot \)m\(^{-1}\)\( \cdot \)s\(^{-1}\)), and \( a \) is the equivalent radius of \( L. \) pneumophila cell (m) (a=0.83 \( \mu \)m). The calculated \( D \) of \( L. \) pneumophila cells is \( 2.92 \times 10^{-13} \)m\(^2\)/s.

**Statistical analysis**

Statistical analysis was conducted for all Sherwood numbers in both fluorescence microscope and CLSM adhesion experiments. For comparing Sherwood numbers obtained from CLSM adhesion experiments and corresponding fluorescence microscope experiments on a certain biofilm surface, t-test (two tails test with unequal variance) was conducted using at least three replicates. In addition, Sherwood numbers at different ionic strengths on a certain biofilm were examined by one-way ANOVA. All one-way ANOVA tests used in this study were conducted with Origin 8, and t-test
was conducted by Microsoft Excel. The significance level of 0.05 was used for both one way ANOVA and t-test.

**Figure A1** Examples of a) fluorescent microscope image and b) CLSM image of adhered *L. pneumophila* cells on 8-week biofilms. The white dots in a) and the green dots in b) are representing the fluorescent *L. pneumophila* cells. The size of a) fluorescent microscope image is 0.395×0.296 mm. The size of b) CLSM image is 0.720×0.720.
Figure A2 Electrophoretic mobilities of *L. pneumophila*, the 2-week biofilm, the 4-week biofilm, the 8-week biofilm, the 14-week biofilm, and the 29-week biofilm as a function of ionic strength at pH 8.2-8.5.
Figure A3 Sherwood numbers of *L. pneumophila* examined in fluorescence microscope adhesion experiments as a function of relative biofilm roughness coefficient at 100 mM.
Figure A4 Detachment of *L. pneumophila* from a rough and a smooth surface under average flow velocity of a) 0.1 m/s, b) 0.3 m/s, and c) 0.7 m/s.
**Figure A5** Shear stress distribution simulation for a rough biofilm under the average flow velocity of a) 0.1 m/s, b) 0.3 m/s, and c) 0.7 m/s. The same simulation was also conducted for a smooth biofilm profile under the average flow velocity of d) 0.1 m/s, e) 0.3 m/s and, f) 0.7 m/s. 0.1, 0.3, and 0.7 m/s are the average flow rate used in detachment experiments.
**Table A1** Thickness and relative roughness coefficients of biofilms used in adhesion experiments.

<table>
<thead>
<tr>
<th>Thickness (μm)</th>
<th>Relative roughness coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-week</td>
<td>20±4</td>
</tr>
<tr>
<td>8-week</td>
<td>33±7</td>
</tr>
<tr>
<td>14-week</td>
<td>38±5</td>
</tr>
<tr>
<td>29-week</td>
<td>32±14</td>
</tr>
</tbody>
</table>

**Table A2** Thickness and relative roughness coefficient of 8-week biofilm under static condition and different average flow velocity.

<table>
<thead>
<tr>
<th>Static condition, Average flow velocity = 0.007 m/s</th>
<th>Average flow velocity = 0.03 m/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm thickness (μm)</td>
<td>51±2</td>
</tr>
<tr>
<td>Biofilm relative roughness coefficient</td>
<td>0.15±0.05</td>
</tr>
</tbody>
</table>

These thickness and relative roughness coefficients were proven to be statistically the same by ANOVA test.
Table A3 Average thickness and relative roughness coefficient of both the rough and smooth biofilms used in detachment experiment. These biofilms were continuously exposed to different flow velocity of 0.1, 0.3 and 0.7 m/s for 30 minutes. Thickness and roughness was quantified at t=0 and t=30 min.

<table>
<thead>
<tr>
<th>Flow velocity</th>
<th>Thickness (μm)</th>
<th>Relative roughness coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t=0</td>
<td>t=30 min</td>
</tr>
<tr>
<td>0.1 m/s</td>
<td>61±13</td>
<td>62±11</td>
</tr>
<tr>
<td>0.3 m/s</td>
<td>69±4</td>
<td>67±9</td>
</tr>
<tr>
<td>0.7 m/s</td>
<td>68±3</td>
<td>69±4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Flow velocity</th>
<th>Thickness (μm)</th>
<th>Relative roughness coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t=0</td>
<td>t=30 min</td>
</tr>
<tr>
<td>0.1 m/s</td>
<td>65±9</td>
<td>67±6</td>
</tr>
<tr>
<td>0.3 m/s</td>
<td>61±11</td>
<td>64±11</td>
</tr>
<tr>
<td>0.7 m/s</td>
<td>66±3</td>
<td>65±5</td>
</tr>
</tbody>
</table>

Table A4 Sh numbers calculated from CLSM and FM, as well as the P value obtained from comparing the CLSM and FM results by t-test.

<table>
<thead>
<tr>
<th>Sh value</th>
<th>2-week biofilm</th>
<th>14-week biofilm</th>
<th>18-week biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLSM</td>
<td>FM</td>
<td>P value</td>
</tr>
<tr>
<td>3 mM</td>
<td>0.04±0.03</td>
<td>0.03±0.006</td>
<td>0.55</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.05±0.03</td>
<td>0.04±0.009</td>
<td>0.41</td>
</tr>
<tr>
<td>100 mM</td>
<td>0.14±0.06</td>
<td>0.19±0.05</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Table A5 Parameters used in particle tracing and shear stress distribution simulation.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Particle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>2 $\mu$m</td>
<td></td>
</tr>
<tr>
<td>Shape</td>
<td>Circle</td>
<td></td>
</tr>
<tr>
<td>Density</td>
<td>1050 kg/m³</td>
<td></td>
</tr>
<tr>
<td>Release frequency</td>
<td>200 particles/s</td>
<td></td>
</tr>
<tr>
<td><strong>Flow</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compressibility</td>
<td>Incompressible flow</td>
<td></td>
</tr>
<tr>
<td>Density</td>
<td>1000 kg/m³</td>
<td></td>
</tr>
<tr>
<td>Dynamic viscosity</td>
<td>0.001 Pa$\cdot$S</td>
<td></td>
</tr>
<tr>
<td>Average inflow velocity used in particle tracing</td>
<td>0.007 m/s</td>
<td></td>
</tr>
<tr>
<td>Average inflow velocity used in shear stress distribution</td>
<td>0.007 m/s</td>
<td>0.1 m/s</td>
</tr>
<tr>
<td>Temperature</td>
<td>293 K</td>
<td></td>
</tr>
</tbody>
</table>
Table A6 Final cell detachment ratio and 90% maximal detachment time for *L. pneumophila* detachment from a rough and smooth biofilm surface under the average flow velocity of 0.1, 0.3, and 0.7 m/s.

<table>
<thead>
<tr>
<th>Average flow velocity (m/s)</th>
<th>Rough biofilm</th>
<th>Smooth biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final cell detachment ratio&lt;sup&gt;1&lt;/sup&gt;</td>
<td>90% maximal detachment time&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1</td>
<td>0.45±0.05</td>
<td>9.82±2.00</td>
</tr>
<tr>
<td>0.3</td>
<td>0.53±0.08</td>
<td>6.61±1.15</td>
</tr>
<tr>
<td>0.7</td>
<td>0.73±0.005</td>
<td>3.31±0.49</td>
</tr>
</tbody>
</table>

Final cell detachment ratio<sup>1</sup>: ratio of the number of total detached cells to the number of total adhered cells. 90% maximal detachment time<sup>2</sup>: time consumed to reach the detachment ratio when 90% of maximal detachment ratio is reached. Sherwood number for adhered *L. pneumophila* cells on these two biofilms are 0.023 and 0.013.
Table A7 Research questions, methods and main findings in this study.

<table>
<thead>
<tr>
<th>Research questions</th>
<th>Methods conducted</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>What factors control the adhesion of <em>L. pneumophila</em> on biofilms?</td>
<td>1. Adhesion of <em>L. pneumophila</em> on biofilms with different ages at different ionic strengths was examined by both fluorescence microscope and CLSM. 2. Biofilm structure was determined using OCT.</td>
<td>1. On clean PVC and young biofilm surfaces, the adhesion was dependent on ionic strength. 2. On mature biofilms, the adhesion was controlled by biofilm surface roughness.</td>
</tr>
<tr>
<td>How does biofilm surface roughness control <em>L. pneumophila</em> adhesion?</td>
<td>Hydrodynamic condition above biofilm with different structure and corresponding particle tracing was simulated by COMSOL.</td>
<td>1. The enlarged biofilm surface area on rougher surface compared to smooth surface enhanced the adhesion of <em>L. pneumophila</em>. 2. The significantly changed direction of flow streamline along the rough biofilm asperities and the low flow zones surrounding the roughness asperities enhanced particle interception on rough surface.</td>
</tr>
<tr>
<td>Does biofilm surface roughness also affect detachment of <em>L. pneumophila</em> from biofilms? Flow?</td>
<td>1 A rough and a smooth biofilm was selected using OCT. 2 Detachment of <em>L. pneumophila</em> from a rough and a smooth biofilm surface at different flow rates was examined by fluorescence microscope. 3 Shear stress distribution above the smooth and rough biofilm surface profile was simulated by COMSOL.</td>
<td>1 For 0.1 and 0.3 m/s flow velocities, detachment from the rough biofilm was lower compared to the smooth biofilm because larger low-shear-stress zones were created on the rough surface. 2 At the flow rate of 0.7 m/s, equally high detachment was found for both smooth and rough surfaces, which may be due to the detached biofilm surface layer.</td>
</tr>
<tr>
<td>When the flow rate changed, how will the detachment of <em>L. pneumophila</em> change?</td>
<td>Shear stress distribution above the biofilm profile at different flow rates was compared.</td>
<td>Higher flow rate facilitated <em>L. pneumophila</em> detachment from a given biofilm, due to the higher shear stress.</td>
</tr>
</tbody>
</table>
References


APPENDIX B: SUPPORTING INFORMATION FOR CHAPTER 3

Sphere Probe Preparation

The sphere probe used in this study was built by adhering a silica sphere into the tipless cantilever (Calibrated Spring Constant=0.6-1.2 N/m, Mikromasch, Lady's Island, SC). The probe building process was carefully performed using an inverted microscope mounted with a micromanipulator. The silica spheres were washed by repeating 3 times the process of dispersing these spheres in DI water, centrifuging, and removing supernatant. Then the spheres were dispersed in DI water again. 100 µl of this sphere solution was dropped on a mica wafer (diameter = 10 mm) and dried in the air to form a monolayer of spheres onto the mica surface. The mica wafer was then placed in a glass slide fixed in the microscope stage. Before the probe making process, a drop of the UV glue was also added on the same glass slide. To avoid probe contamination caused by directly dipping the tipless cantilever to the large area of UV glue, a waste AFM tip was used to dip in the UV glue and touch on the clean glass slide area several times to create a very small area of glue drop using the micromanipulator. In the probe making process, the micromanipulator moved the tipless cantilever to the small glue drop area and carefully daubed a very small amount of glue to the very tip of cantilever. After that, the cantilever moved on top of the mica wafer and adhered one sphere using the micromanipulator. The UV glue between the cantilever and the sphere was cured under UV light for one hour. All the AFM sphere probes were cleaned in an UV-Ozone generator for one hour.

Examination of AFM Probe Contamination

The contamination of the AFM probe was checked by measuring a normal force-distance curve on a clean glass slide in water. If the AFM probe would be contaminated by residual biofilm, the retraction force curve would exhibit adhesion (Figure S5a). When this occurred, a new probe was
used. If no adhesion force was observed in the retraction force curve (Figure S5b), the probe was considered to be suitable for further use. Each AFM probe was typically used to conduct approximately 100-500 indentation measurements before contamination was detected.

**Statistical analysis**

OriginPro 9.0 (OriginLab Corporation, Northampton, MA) was used for statistical analysis of the AFM, OCT, and CLSM results. For comparing the biofilm Young’s modulus distributions obtained by AFM, Kolmogorov-Smirnov tests were conducted between two examined distributions. For biofilm thickness and roughness results obtained by OCT, t-tests were used to compare the biofilm structures at different times or under different treatment conditions. T-tests were also conducted for comparing protein/polysaccharide ratios obtained by CLSM.
Figure B1 a) The percentage stacked bar and b) the outer layer thickness of biofilms during the 3 months of free chlorine treatment under stirring condition. The red line in Figure a) shows the mean value of $E$ at each time point.
Figure B2 a) The percentage stacked bar and b) the outer layer thickness of biofilms during the 3 months of monochloramine treatment under no stirring condition. The red line in Figure a) shows the mean value of $E$ at each time point.
Figure B3 a) The percentage stacked bar and b) the outer layer thickness of biofilms during the 3 months of free chlorine treatment under no stirring condition. The red line in Figure a) shows the mean value of $E$ at each time point.
Figure B4 a) Average thickness and b) relative roughness coefficient of biofilms after 3 months of exposure to monochloramine, free chlorine, and groundwater without disinfectant under shearing and no shearing conditions, respectively. The average thickness and roughness were calculated from 20 randomly selected OCT images.
Figure B5 Calibration force-distance curves obtained in water on clean glass surfaces showing a) probe contamination and b) clean probe without contamination.
Table B1 Change of biofilm average thickness during the disinfectant exposure under different treatment

<table>
<thead>
<tr>
<th>Exposure time</th>
<th>Monochloramine + Shear</th>
<th>Free Chlorine + Shear</th>
<th>Groundwater + Shear</th>
<th>Monochloramine + Shear-free</th>
<th>Free Chlorine + Shear-free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>120±8</td>
<td>120±8</td>
<td>120±8</td>
<td>120±8</td>
<td>120±8</td>
</tr>
<tr>
<td>Week 2</td>
<td>119±6</td>
<td>114±12</td>
<td>116±12</td>
<td>111±15</td>
<td>117±9</td>
</tr>
<tr>
<td>Week 3</td>
<td>110±10</td>
<td>113±8</td>
<td>114±7</td>
<td>107±8</td>
<td>110±15</td>
</tr>
<tr>
<td>Week 4</td>
<td>108±6</td>
<td>107±11</td>
<td>125±11</td>
<td>100±5</td>
<td>103±8</td>
</tr>
<tr>
<td>Week 5</td>
<td>105±6</td>
<td>131±9</td>
<td>124±11</td>
<td>93±6</td>
<td>116±10</td>
</tr>
<tr>
<td>Week 7</td>
<td>120±7</td>
<td>139±8</td>
<td>111±16</td>
<td>105±8</td>
<td>116±8</td>
</tr>
<tr>
<td>Week 9</td>
<td>118±8</td>
<td>135±9</td>
<td>112±11</td>
<td>95±14</td>
<td>113±11</td>
</tr>
<tr>
<td>Week 10</td>
<td>130±8</td>
<td>130±12</td>
<td>124±12</td>
<td>98±7</td>
<td>105±9</td>
</tr>
<tr>
<td>Week 11</td>
<td>130±9</td>
<td>132±11</td>
<td>133±14</td>
<td>109±6</td>
<td>105±9</td>
</tr>
<tr>
<td>Week 13</td>
<td>129±8</td>
<td>127±19</td>
<td>123±18</td>
<td>110±7</td>
<td>117±6</td>
</tr>
</tbody>
</table>

* The average thickness and standard deviation was calculated from analysis on 20 randomly selected OCT images.
Table B2 Change of biofilm relative roughness coefficient during the disinfectant exposure under different treatment

* The average roughness and standard deviation was calculated from analysis on 20 randomly selected OCT images.

<table>
<thead>
<tr>
<th>Exposure time</th>
<th>Monochloramine + Shear</th>
<th>Free chlorine + Shear</th>
<th>Groundwater + Shear</th>
<th>Monochloramine + Shear-free</th>
<th>Free chlorine + Shear-free</th>
<th>Groundwater + Shear-free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
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Antibiotic concentration determination for *L. pneumophila*

The *L. pneumophila* cells used in our study carried a chloramphenicol resistance plasmid. To determine the concentration of culturable *L. pneumophila* (CFU/mL) released from biofilms, the released sample was cultured in the buffered charcoal yeast extract (BCYE) agar plates with the chloramphenicol concentration of 10 µg/L. To ensure that this chloramphenicol concentration won’t inhibit the growth of *L. pneumophila*, the growth of pure culture *L. pneumophila* at the chloramphenicol concentration of 0, 5, 7.5, 10, 15, and 20 µg/L was examined. A serial 10-fold dilutions of *L. pneumophila* solutions were prepared and plated on the agar medium with different chloramphenicol concentration. After incubating these plates at 37°C, the CFU in each plate was counted. The concentration of *L. pneumophila* at each chloramphenicol level was 1.55×10⁷, 1.43×10⁷, 1.39×10⁷, 1.40×10⁷, 1.42×10⁷, and 1.22×10⁷ #/mL at 0, 5, 7.5, 10, 15, and 20 µg/L chloramphenicol, respectively. The *L. pneumophila* concentrations at 0, 5, 7.5, 10, and 15 µg/L chloramphenicol were statistically the same, suggesting that chloramphenicol level lower than 15 µg/L won’t inhibit the growth of *L. pneumophila*.

To ensure that 10 µg/L of chloramphenicol can prevent the growth of other bacteria, *L. pneumophila* liquid culture was mixed with *Staphylococcus epidermidis* culture (1:1) and plated on BCYE plates with different concentration of 0, 5, 7.5, 10, 15, and 20 µg/L. The *L. pneumophila* strain was green florescence protein (GFP) tagged and would show green colonies on BCYE plates, while *Staphylococcus epidermidis* showed white colonies on BCYE plates. Therefore, the *L. pneumophila* colonies could be distinguished from the *Staphylococcus epidermidis* colonies. The concentration of *L. pneumophila* at each chloramphenicol level was 7.20×10⁷, 6.80×10⁷, 7.70×10⁷,
7.80×10^7, 6.33×10^7, and 6.15×10^7 #/mL at 0, 5, 7.5, 10, 15, and 20 µg/L chloramphenicol, respectively. The *Staphylococcus epidermidis* colonies were only observed at the concentration at 0, 5, and 7.5 µg/L chloramphenicol. The concentration of *Staphylococcus epidermidis* was 8.00×10^7, 9.50×10^7, and 2.9×10^7 at 0, 5, and 7.5 µg/L chloramphenicol, respectively. Therefore, at 10 µg/L chloramphenicol the *Staphylococcus epidermidis* did not grow and *L. pneumophila* won’t be inhibited.

**The possible change of *L. pneumophila* cultivability with time after disinfection**

In this study, the plate counting assay was conducted immediately after the *L. pneumophila* release experiments. Previous study showed that some bacteria cells injured by disinfection may recover after staying in disinfectant-free condition for some time.29 Therefore, to ensure the plate counting results of released *L. pneumophila* in this study did not increase with time, control experiments were conducted to determine the concentration of culturable *L. pneumophila* at 0, 1, and 2 days after disinfection.

1 ml of 10^8 #/mL *L. pneumophila* was prepared and added to 100 mL groundwater containing free chlorine of 0.5 Cl₂ mg/L. 3 mL of *L. pneumophila* sample was then taken after 1 min and 5 min of disinfection at stirring condition, respectively, and immediately mixed with 30 µL 5% sodium thiosulfate. The concentration of these two *L. pneumophila* samples were determined by the serial 10-fold dilution plate counting at Day 0, Day 1, and Day 2. The concentration of *L. pneumophila* sampled at 1 min of disinfection was 8.09×10^7, 6.50×10^7, and 6.35×10^7 #/mL at Day 0, Day 1, and Day 2, respectively. The concentration of *L. pneumophila* sampled at 5 min of disinfection was 750, 700, and 100 #/mL at Day 0, Day 1, and Day 2. These results suggested that the number of culturable *L. pneumophila* did not increase with time after disinfection.
Figure C1 qPCR inhibitor testing curve showing the linear correlation between the \textit{L. pneumophila} copy number concentration (qPCR results) and dilution ration (1/dilution times). The good linear correlation revealed that the qPCR process was not affected by the possible inhibitors in the extracted DNA samples.

Figure C2 Linear correlation between qPCR measurement results and CFU counting results.
Figure C3 Inactivation ratio of *L. pneumophila* released from groundwater (GW) biofilms and monochloramine-treated biofilms by monochloramine. The inactivation ratio was defined by the ratio of non-cultivable *L. pneumophila* to total released *L. pneumophila*.

Figure C4 Infectivity of *L. pneumophila* released from groundwater (GW) biofilms and free chlorine-treated biofilms by free chlorine contained groundwater. The infectivity was defined by the ratio of *L. pneumophila* population after infecting amoeba to *L. pneumophila* population before infecting amoeba.