ROLE OF DISINFECTANTS AND PIPE MATERIALS ON BACTERIAL ADHESION ONTO BIOFILMS

BY
DAO SUWANSANG JANJAROEN

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environmental Engineering in Civil Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Doctoral Committee:

Associate Professor Thanh Huong Nguyen, Chair
Professor Wen-Tso Liu, Co-Chair
Professor Benito Jose Mariñas
Professor Eberhard Morgenroth
Professor Nicholas Ashbolt
ABSTRACT

Biofilms are ubiquitous in aquatic environments. Biofilms have been shown to attract and harbor pathogens such as *P. aeruginosa* and *Legionella pneumophila* in premise plumbing system. The fact that biofilms can protect attached bacterial cells from disinfectants raises rudimentary questions regarding interactions of bacterial cells with biofilm surfaces. Consequently, the main objectives of this study were to: 1) investigate the mechanisms that govern *E. coli* S17, *E. coli* 14f and *Legionella* cells adhesion on clean PVC, copper and biofilms; 2) examine the role of disinfectants on biofilms structure and subsequent effect on bacterial adhesion.

Mechanisms of three strains of bacteria attachment on biofilms grown on PVC and copper surfaces were investigated. Biofilms were grown in CDC reactors using different types of feed water such as groundwater, monochloramine-treated groundwater, dechlorinated tap water and tap water. Biofilm physical structure was characterized at micro- and meso-scales using Scanning Electron Microscopy, Optical Coherence Tomography and Confocal Laser Scanning Microscopy. On clean PVC, copper and biofilms surfaces, the adhesion of three bacterial strains was found to increase as a function of ionic strength. However, on established biofilms, the adhesion was independent of solution chemistry. It rather had a positive correlation with biofilm roughness. Adhesion of every bacterial strain had found to increase on rougher biofilms than smoother ones.

Besides normally grown biofilms, disinfectants were also introduced into feed water. After 3 months of exposure to monochloramine, aged groundwater biofilms...
became smoother. This smooth biofilm surface discouraged bacterial cells from adhering. Even though disinfectant can alter biofilm surface roughness, it did not seem to change the average thickness of well-established biofilms. Besides monochloramine, free chlorine from tap water was able to eradicate thin biofilms from the pipe surface leading to lower adhesion of bacterial cells.

The effect of bacteria surface hydrophobicity on bacterial adhesion was also investigated by starving *Legionella* cells in Newmark Groundwater. Starved cells exhibited more hydrophobicity and adhered more on hydrophobic PVC surfaces than fresh cells. Conversely, adhesion of starved cells on copper surfaces was lower than fresh cells due to incompatibility of hydrophobicity between bacterial cells and copper surfaces.
This thesis is dedicated to my Mom, Dad, Brothers and Kenneth for their love and support throughout these years.
ACKNOWLEDGEMENTS

I would like to thank many people who have helped me through the completion of this dissertation. The first is my adviser, Prof. Helen Nguyen, who is very patient, supportive, knowledgeable, encouraging, and the true embodiment of a mentor. Without her, I wouldn't have made it this far. Thank you for making me push myself harder. In combination with mentorship from my adviser, I am very blessed to work with intelligent and proficient committee members; Prof. Benito Jose Mariñas, Prof. Wentso Liu, Prof. Eberhard Morgenroth, and Dr. Nicholas Ashbolt.

I also would like to thank Prof. Vern Snoeyink for his encouragement, support and insightful discussion during my experimental work. Also, working with Dr. Nicolas Derlon and Guillermo Monroy is an invaluable experience. Their expertise encourages me to expand my knowledge into different fields. I would like to show my gratitude to brilliant technicians that I have been trained with; Dr. Scott Maclaren, Dianwen Zhang, Scott Robinson, Dr. Christopher B. Huppenbauer and Steve Pickens. Without them, my result section wouldn't be complete. I also thank you Dr. Shaoying Qi for his knowledge and discussion about my research.

This work was not completed in a vacuum. I am grateful to work and share my life with descent colleagues whom I can call life-time friends; Leonardo Gutierrez, Yuanyuan Liu, Yun Shen, Nanxi Lv, Ofelia Romero, Francisco Mena, Susana Kimura, Fangqiong Ling, Ya Zhang, Andres Jurado, Courtney Flores and Hanting Wang. Working with these friends has been a gift and expands a value of work. I can't feel
more fortunate to have them in my life. I also want to thank my undergraduate assistant, Lance Langer, who helped me maintain my biofilm reactors.

I am thankful and would like to acknowledge many others who helped me along the way, my mom and my dad who gave unconditional love for me during my busy time, my brothers who supported all decisions I made, my housemates who cooked for me during my thesis writing, my nanny who helped take care my dogs at home. My gym companion, Pimonrat Tiansawat, who shared common interest with me.

There are also many neglected people and groups that are involved in the completion of my Ph.D. that I would like to acknowledge. I would like to thank Mary Pearson and Joan Christian for their coordination for my Ph.D. defense arrangement. I also would like to thank Grainger Engineering Library and Urbana Public Library for providing me such a great environment to write my thesis.

Kenneth Ng, my fiancé, thank you for being supportive and proofing my thesis and manuscript.

I also would like to acknowledge Royal Thai Government Scholarship for supporting my study in the U.S., the WaterCAMPWS who sent me to many conferences, and EPA grant who funded my research. Without these organization, I wouldn’t have been here.

The path to becoming a doctor makes me a better version of myself. I’d like to thank everyone involving in this process for making me the person I am.
# TABLE OF CONTENTS

CHAPTER 1 – INTRODUCTION ........................................................................................................ 1

CHAPTER 2 – ROLE OF IONIC STRENGTH AND BIOFILM ROUGHNESS ON DEPOSITION KINETICS OF *ESCHERICHIA COLI* ONTO GROUNDWATER BIOFILM GROWN ON PVC SURFACES .......................................................................................... 10

CHAPTER 3 – ROLE OF DISINFECTANTS ON ADHESION KINETICS OF *ESCHERICHIA COLI* AND *LEGIONELLA PNEUMOPHILA* ONTO GROUNDWATER AND TAP WATER BIOFILM GROWN ON PVC SURFACES .......................................................... 52

CHAPTER 4 – THE EFFECT OF CELL STARVATION ON ADHESION KINETICS OF *LEGIONELLA PNEUMOPHILA* ONTO PVC, COPPER AND BIOFILMS .................................................. 89

CHAPTER 5 – CONCLUSIONS AND FUTURE RESEARCH .......................................................... 113

APPENDIX – PERMISSION LETTER FROM WATER RESEARCH .................................................. 117
CHAPTER 1

INTRODUCTION

1.1. Background

Despite perpetual advances in water treatment technologies, drinking waterborne outbreaks continue to occur around the world. In the United States from 1920 to 1990, 11-18% of reported waterborne disease outbreaks were associated with contamination of drinking water distribution systems (DWDS) (Craun et al., 1997, 2006). In 2009-2010, 33 drinking water-associated outbreaks were reported, resulting in 1,040 cases of illness, 85 hospitalizations, and 9 deaths (Hilborn et al., 2013). Several factors may contribute to the contamination of drinking water in distribution systems. Problems with the physical integrity of DWDS, cross connections, and intermittent pressures can lead to intrusion of pathogens into the system (Snoeyink et al., 2006).

Residual disinfectants are required in DWDS to ensure the integrity of finished drinking water in the system (Fraser et al., 1977). However, the quality of drinking water in the system can be degraded by the presence of biofilms by two reasons (Kim et al., 2002; Niquette et al., 2000; Norton and LeChevallier, 1997; Snoeyink et al., 2006; Szewzyk et al., 2000). First, biofilms in DWDS have been found to attract, protect, and harbor pathogens such as Legionella pneumophila and Mycobacterium avium (Berry et al., 2006; Lau and Ashbolt, 2009; Norton et al., 2004; Wullings et al., 2011). Second, pathogenic problems are worsened as the consumption of residual disinfectants by biofilms in these systems lead to disinfectant deficiency (Xue and Seo, 2013; Xue et al.,
2012), especially in premise plumbing systems where the amount of residual chlorine is not regulated (Fraser et al., 1977).

The presence of biofilms in premise plumbing raises fundamental questions regarding the interaction mechanisms between biofilms, disinfectants, and bacterial pathogens that may affect transport of pathogens in DWDS. The way in which biofilms attract and allow adherence of different types of cells and material particles to biofilms has been widely studied. Biofilm surface roughness, solution chemistry, and cell surface hydrophobicity have all been identified to influence cell or particle adhesion. Both E. coli cells and polystyrene spheres adhere more on biofilms grown from tap water than on a clean glass slide (Paris et al., 2009), while Cryptosporidium oocysts deposition shows that biofilm roughness control the adhesion of oocysts to Pseudomonas aeruginosa biofilms (Searcy et al., 2006; Wu et al., 2012). Studies (Paris et al., 2009; Searcy et al., 2006) which found surface roughness to influence particle adhesion have also provided information on particle interaction with different surface structures. Abiotic surfaces such as glass and rock have also been shown to retain more particles with higher surface roughness. The Sherwood number of polystyrene latex beads has been found to be higher on rough rock surfaces (Darbha et al., 2012). The effect of solution chemistry on particle adhesion is also well studied (Elimelech et al., 1998; Janjaroen et al., 2010; Liu et al., 2009; Redman et al., 2004; Song and Elimelech, 1995; Walker et al., 2005). Adhesion of bacterial cells on glass surface has been shown to increase with ionic strength of the solution (Liu et al., 2009; Redman et al., 2004) due to the compression of cell double layer thickness. In addition to solution ionic strength, cell surface hydrophobicity also plays an important role in bacterial adhesion (Liu et al., 2004;
Vanloosdrecht et al., 1987; Zita and Hermansson, 1997). Specifically, starvation of bacterial cells may lead to changes in surface hydrophobicity which subsequently affect bacterial attachment on a surface (Husmark and Rönner, 1992; van Loosdrecht et al., 1987). Moreover, studies have suggested that pipe materials have an influence on bacterial adhesion (Niquette et al., 2000; Simões et al., 2007).

Consequently, the abovementioned studies give us an initial understanding of how surface roughness or chemistry affect the interaction between cells or particles, and biofilms. Unfortunately, most of the studies mentioned above focused on engineered biofilms which were grown with known bacteria. There has been limited research on biofilms grown from non-laboratory bacteria, especially bacteria from drinking water. Also, bacteria which have been used in adhesion experiments were not diverse. As a result, further studies are essential in order to understand biofilm-bacteria interaction as well as the role of chemical disinfectants on bacterial attachment to biofilms.

1.2. Objectives

The main objectives of this study were to
- Develop a methodology to study the adhesion of 3 different bacterial strains (laboratory *E. coli* S17, environmental *E. coli* 14f and *Legionella pneumophila*) on groundwater and tap water biofilms. This system represents the fate and transport of bacteria in DWDS.
- Elucidate the effect of surface roughness on bacterial adhesion. Varying biofilm roughness, with controls included, was achieved with different biofilm growth time and disinfectants. The adhesion of three bacterial strains was conducted on different
biofilm roughness to study the influence of biofilm roughness on cell adhesion. Two main techniques (optical coherence tomography and confocal laser scanning microscopy) were used to measure biofilm surface roughness depending on biofilm thickness.

- Study the effect of solution chemistry on bacterial adhesion on piping of different materials (e.g. PVC and copper) as well as on biofilms and disinfected biofilms. This objective mainly focused on a monovalent salt solution (KCl) at pH 8.2 to mimic the environment in DWDS.

- Examine the role of bacterial cell surface hydrophobicity on cell adhesion on different types of surfaces (e.g. PVC, copper and biofilms). Bacterial cells were subjected to starvation in low nutrient environments to change the cells’ surface hydrophobicity. Microbial adhesion to hydrocarbon (MATH) test was conducted for each bacterial type for hydrophobicity.

1.3. **Experimental Approach**

Biofilms were grown on PVC and copper surfaces in well controlled environments using groundwater and tap water as feeds. Biofilms were grown to different ages and were subjected to different types of disinfectants (chlorine and monochloramine). Adhesion of three bacterial strains (laboratory *E. coli* S17, environmental *E. coli* 14f and *Legionella pneumophila*) on various surfaces was studied *ex-situ* in a parallel plate flow chamber (PPFC). Adhesion experiments were performed at different KCl concentrations ranging from 3-300 mM at pH 8.2-8.5. Adhered bacterial cells on biofilms were observed with 2 different microscopy techniques: epifluoresence microscopy and confocal laser scanning microscopy (CLMS). Biofilm surface roughness and total thickness were measured by optical coherence tomography (OCT) and CLSM.
Moreover, hydrophobicity and surface charge were analyzed by MATH test and
dynamic light scattering (DLS), respectively. Scanning electron microscopy (SEM) was
used to image biofilms. For PVC and copper surfaces, roughness was measured with a
profilometer. A detailed description of the aforementioned techniques is described in
CHAPTER 2, 3 and 4.

1.4. References

Berry, D., Xi, C., Raskin, L., 2006. Microbial ecology of drinking water distribution

Chen, X., Stewart, P.S., 1996. Chlorine Penetration into Artificial Biofilm is


Aggregation: Measurement, Modelling and Simulation (Colloid & surface engineering).
Buttenworth-Heinemann.

Fraser, D.W., Tsai, T.R., Orenstein, W., Parkin, W.E., Beecham, H.J., Sharrar,
R.G., Harris, J., Mallison, G.F., Martin, S.M., McDade, J.E., Shepard, C.C., Brachman,
Med 297, 1189–1197.


CHAPTER 2

ROLE OF IONIC STRENGTH AND BIOFILM ROUGHNESS ON DEPOSITION KINETICS OF ESCHERICHIA COLI ONTO GROUNDWATER BIOFILM GROWN ON PVC SURFACES

2.1. Abstract

Mechanisms of *Escherichia coli* attachment on biofilms grown on PVC coupons were investigated. Biofilms were grown in CDC reactors using groundwater as feed solution over a period up to 27 weeks. Biofilm physical structure was characterized at the micro- and meso-scales using Scanning Electron Microscopy (SEM) and Optical Coherence Tomography (OCT), respectively. Microbial community diversity was analyzed with Terminal Restricted Fragment Length Polymorphism (TRFLP). Both physical structure and microbial community diversity of the biofilms were shown to be changing from 2 weeks to 14 weeks, and became relatively stable after 16 weeks. A parallel plate flow chamber coupled with an inverted fluorescent microscope was also used to monitor the attachment of fluorescent microspheres and *E. coli* on clean PVC surfaces and biofilms grown on PVC surfaces for different ages. Two mechanisms of *E. coli* attachment were identified. The deposition rate coefficients ($k_d$) of *E. coli* on nascent PVC surfaces and 2-week biofilms increased with ionic strength. However, after biofilms grew for 8 weeks, the deposition was found to be independent of solution chemistry. Instead, a positive correlation between $k_d$ and biofilm roughness as determined by OCT

was obtained, indicating that the physical structure of biofilms could play an important role in facilitating the deposition of *E. coli* cells.

### 2.2. Introduction

Biofilms are aggregates of cells and extracellular polymeric substances (EPS), and are found ubiquitously in both natural and engineered systems, such as on a pipe surface in Drinking Water Distribution Systems (DWDS) (Berry et al. 2006, Flemming and Wingender 2010). Biofilms in DWDS were reported to be capable of attracting and harboring pathogens (Berry et al. 2006). In addition, biofilm matrix may prevent disinfectants from reaching the cells located deep inside the biofilm (Berry et al. 2009, Gagnon et al. 2008, Norton et al. 2004, Williams and Braun-Howland 2003). As a result, pathogenic microorganisms such as *Mycobacterium avium* and *Legionella pneumophila* have been found in DWDS biofilms (Declerck et al. 2009, Falkinham et al. 2001, Lau and Ashbolt 2009, Le Dantec et al. 2002, Torvinen et al. 2004, Valster et al. 2011, Wullings et al. 2011). More importantly, pathogen presence and survival in DWDS has been linked to outbreaks (Craun et al. 2010, 1998, 2002). Thus, understanding the mechanisms of pathogen attachment to biofilms developed in DWDS is of crucial interest to ensure the quality of the drinking water.

While previous studies have convincingly presented the evidence that biofilms can harbor pathogens (Altman et al. 2009, Flemming and Wingender 2010, Helmi et al. 2010, Helmi et al. 2008, Kumar and Anand 1998), systematic studies to identify the physical and chemical factors controlling pathogen attachment to biofilm are rare. For example, *Escherichia coli* and fluorescent polystyrene beads have been found to attach more to biofilms grown from tap water on glass slides than to the surface of clean glass
slides (Paris et al. 2009). Biofilm characteristics such as age and coverage have been identified as controlling factors for *E. coli* and microsphere attachment (Paris et al. 2007, 2009). In contrast, attachment of *Legionella*, bacteriophages, and microspheres on biofilms grown from lake water on glass surface was found to be independent of biofilm cell surface density, but dependent on particle surface properties, such as hydrophobicity (Långmark et al. 2005). Spatial distribution of biofilms but not their cell density was found to be dependent on the wall shear rate (Paris et al. 2007). It is likely that under different shear rates, biofilms can develop into different physical structures. However, the role of biofilm physical structure on bacterial cell attachment to biofilms has not been investigated.

Our study aims to elucidate the mechanisms that govern the attachment of *E. coli* S17 to groundwater biofilms grown on PVC surfaces. Specifically, we will focus on the role of water chemistry and biofilm structure on *E. coli* attachment. We use *E. coli* S17 as a surrogate of bacterial pathogens because deposition of *E. coli* to the biofilms represents intrusion of biological contaminants into DWDS. The advantages of using groundwater, which is the source for drinking water in the Champaign-Urbana area, include a stable chemistry, disinfectant free and higher carbon source, allowing faster biofilm growth. A parallel plate flow chamber (PPFC) was used to monitor attachment of *E. coli* onto clean PVC surfaces and biofilm grown on PVC. Physical and biological characterization of groundwater biofilm was used to explain attachment mechanisms.

2.3. **Materials and Methods**

**Bacteria Cell Preparation.** *Escherichia coli* (*E. coli* S17-1 λ-pir) was obtained from Dr. Thomas at the University of Wisconsin (Simon et al. 1983). This *E. coli* was
tagged with Green Fluorescing Protein (GFP) plasmid. For the deposition experiment, a single colony was picked from a freshly prepared plate and pre-cultured in Luria-Bertani (LB) broth with 50 µg/L carbenicillin at 37 °C overnight with 200 rpm shaking. This preculture was diluted 100 times in fresh LB media containing carbenicillin and incubated for 8 hours until reaching an optical density of 0.8 at 600 nm (OD_{600}). Then, 1 mM IPTG was added to the stock to induce fluorescent expression, and the stock was incubated for 2 more hours to get to 1.2 OD_{600}. After 10 hours of incubation, cells were harvested by centrifugation at 17000 × g, cleaned by suspending in 10 mM KCl buffered with 1mM NaHCO₃, followed by centrifugation. The cleaning and centrifugation steps were repeated twice. Freshly grown *E. coli* were tested with viability tests using Live/Dead BacLight kit (Invitrogen L7012) in experimental ionic strength (IS) KCl solutions. The stained cells were directly counted under an inverted fluorescent microscope (DM15000 M, Leica, Wetzlar, Germany) with the suitable fluorescent filter set (Chroma Technology Corp.). Phase contrast images of *E. coli* cells were compared with fluorescence images of this same sample using a microscope with an oil objective at 63X magnification to ensure that all cells were emitting fluorescence.

**Biofilm Preparation.** A CDC reactor (CBR 90-2) was obtained from BioSurface Technologies Corporation and was used to grow biofilm on PVC coupons (RD 128-PVC). PVC coupons were secured to plastic rods in the reactor. Groundwater collected from a natural aquifer underneath the Newmark Civil Engineering Laboratory (205 N. Matthews, Urbana, Illinois, 61801) was first treated with a greensand filter to remove iron and manganese. This groundwater was well characterized and used in previous studies (Bradley et al. 2011, Li et al. 2002). The chemical characteristics of the
groundwater including alkalinity, hardness and trace metals, were analyzed by the Illinois State Water Survey. Groundwater was collected into a reservoir every 2 days and was continuously pumped through the reactor at a flow rate of 1.30 mL/min corresponding to a hydraulic retention time of 4 hours. Mixing of the bulk liquid was performed using a magnetic stirrer at 125 rpm. Biofilms were grown to different ages from 2 to 27 weeks.

**Contact Angle Measurement and Surface Energy Estimation.** Contact angle measurements of *E. coli*, biofilm, and PVC were measured by static sessile drop technique using a Goniometer (KSV Instrument, CAM 200). Diiodomethane, which is non-polar and hydrophobic, was used as a probe liquid in contact angle measurements. The contact angle between diiodomethane and the surface was used to calculate the Lifshitz–van der Waals (γ_LW) component of surface energy (Brant and Childress 2002, Busscher et al. 1984, van der Mei et al. 1998, van Oss 1993, Zaidi et al. 2011). A layer of *E. coli* cells was captured on a membrane surface by filtering the cell suspension through a 0.45 μm membrane filter (Whatman 7184-004). The *E. coli* cell concentration on the filter was 10⁸ cells/cm². This filter was kept on top of a 10% agar plate, containing 20% glycerol, to keep the cell lawn hydrated. The filters with *E. coli* lawn and the coupons from the CDC reactor were left undisturbed in a covered petri dish for 10-20 min before the contact angles measurements. This period of time was necessary to transfer the samples from the reactors and the media to the goniometer setup. The samples subjected to contact angle measurement were fully saturated with water and were not suitable for being probed with a water drop. Five microliters of diiodomethane was dropped on each surface, and contact angles were measured immediately for 10
seconds. Left and right contact angles for each surface in at least 3 locations were measured at least 12 times, with highest and lowest values discarded. The equilibrium contact angle was calculated as the average of each side contact angle.

A control experiment of contact angles was done on 24-week old biofilms at different drying times (from 0 to 120 min) (Busscher et al. 1984, van der Mei et al. 1998). As shown in Figure 2.1, contact angle was the largest at 0 min and stabilized after 30 min of measurement. At time 0, we suspected that the contact angle was likely measured on a water layer instead of hydrated biofilm. Because the contact angle was consistent for 30 min up to 120 min of drying time, we used a 30 min drying time for all measurements. This protocol is similar to the one used in Park and Abu-Lail (2011). Contact angle using water was attempted on the biofilm, however, the water drop was quickly absorbed by the biofilm to prevent consistent measurement. Following the work by Park and Abu-Lail (2011), we obtained Atomic Force Microscopy (AFM) images of the well-dispersed *E. coli* deposited on a glass surface to show that the height of *E. coli* cells was 600 nm (Fig. 2.2). High concentration of *E. coli* cells on the filter for the contact angle measurement caused the cells to aggregate and cover the entire surface of the filter (Fig. 2.3). The roughness of this *E. coli* lawn on the filter cannot be measured with AFM and OCT imaging. However, this roughness should be smaller than the width of *E. coli* cells, i.e., 600nm. Thus, the roughness of the *E. coli* lawn is much smaller than the size of the drop of contact angle probe liquid and should not interfere with contact angle measurements.
Figure 2.1. Contact angle of diiodomethane on 24-week biofilm as a function of drying time.

Figure 2.2. AFM images for *E. coli* cells on a glass slide. All image analysis was done in liquid environment. All measurements were accomplished in a contact mode using a silicon cantilever (Budget Sensors SiNi-30). Images were taken with a small scanning area (2.5×2.5 μm² or 5×5 μm²) at a scan rate of 10 Hz and with 256 points per scan line.
Figure 2.3. Image of *E. coli* cells lawn on a 0.45 μm membrane filter for contact angle measurement. This filter was kept on top of a 10% agar plate, containing 20% glycerol, to keep the cell lawn hydrated.

The Lifshitz–van der Waals (γ_{LW}) component of surface energy was derived from the contact angles using equation 4 (van Oss 1993). The LW component of free energy of adhesion (ΔG_{yo}^{LW}) between the *E. coli* and biofilm/PVC surface in the presence of water was calculated using equation 2 in Liu et al. (2010). The Hamaker constant (A) was deduced from the LW component of free energy of adhesion (ΔG_{yo}^{LW}) as described in van Oss (1993).

**Electrophoretic Mobility Measurement.** Electrophoretic mobilities (EM) of *E. coli* S17 and biofilm were measured by a Zetasizer Nano ZS90 instrument (Malvern Instruments, Southborough, MA) in various salt concentrations at 25 °C. Electrophoretic mobilities were converted into surface potentials via the Hemholtz-Smoluchowski equation. An *E. coli* concentration of 3 × 10^6 *E. coli/mL in each desired electrolyte solution buffered with 1 mM NaHCO_3 at pH 8.2-8.4 was used in electrophoretic mobility measurements. For biofilm, a PVC coupon from a CDC reactor was sonicated in 5 mL of a given salt concentration at pH 8.2-8.4 for 5 min. Six-week biofilms were sonicated for either 5 min or 30 min to assess the effect of sonication time on EM measurement.
Supernatant was taken to measure EM. At least 3 replicates were conducted for each condition.

**Deposition Experiment.** Deposition of *E. coli* cells on biofilm and PVC surface was studied *ex-situ* in a PPFC (Biosurface Technologies Corp. FC 71). Deposition experiments were performed at different monovalent concentrations (3-300 mM KCl) at 4 × 10^6 *E. coli* cells/mL with a constant flow of 1 ml/min. Electrolyte solutions were buffered at pH 8.2-8.4 by 1 mM sodium bicarbonate. The concentration of *E. coli* cells was selected to ensure that enough deposition could be visualized, and no aggregation was observed during the deposition experiment. Deposited *E. coli* cells were counted with a 40× objective in a rectangular viewing area of 296 × 222 μm² under an inverted fluorescent microscope (Leica DM15000 M) every 15 s for 30 min. The microscope images were recorded by a QIMAGING RETIGA 2000R Fast 1394 camera and were processed by ImagePro 7.0 software. At the end of each experiment, the flow chamber was flushed with 1 mM KCl at the same flow rate for observation of possible cell detachment. Before the deposition experiments, two control experiments were conducted. The first control experiment involved observing *E. coli* cells under a static no-flow condition on a glass slide using phase contrast and then fluorescence. The second control experiment involved observing *E. coli* cells under a continuous flow condition in a PPFC, for which glass coupons were used instead of biofilms so that the cells could be observed using a bright field and then fluorescence. The results of both control experiments confirmed that all *E. coli* cells were fluorescent and can be observed under both static and flow conditions.
**Flow Profile for Parallel Flow Chamber.** The parallel flow chamber used in our deposition experiment had an inlet diameter of 1.45 mm (Biosurface Technologies Corp. FC 71-PC-2×0.5). The width and the length of the flow channel were 13 mm and 39.3 mm, respectively. The depth of the flow cell was 0.39 mm. Inside the flow cell there were 2 slots to attach 2 PVC coupons; however, only 1 slot was used at a time in deposition experiment. The flow velocity profile was calculated by solving the Navier-Stokes equation using finite element algorithm in the software package COMSOL. The solution for the velocity profile was used to select a uniform laminar flow condition inside the parallel flow chamber. The deposition was conducted at Re of 1.24, Pe of 0.3, and shear rate of 35 sec⁻¹.

**Deposition Rate Coefficient Calculation and Statistical Analysis.** The deposition rate coefficient, \( k_d \), was \( E. coli \) deposition flux (number of deposited \( E. coli \) cells per viewing area per time) divided by initial \( E. coli \) cell concentration. Each condition was conducted twice within the same day with the same biofilms taken from the reactor to ensure consistency. Linear regression analysis was used to calculate the \( k_d \) value and the corresponding 95% confidence interval for data obtained for a given condition. \( k_d \) for each biofilm age was plotted as a function of ionic strength. A multiple linear regression analysis (Neter et al. 1990) was used to compare if the slopes of \( k_d \) versus ionic strength of each biofilm age were significantly different (\( p < 0.05 \)) from each other. Only one \( k_d \) value was plotted in the graph. However, the deposition experiment was conducted twice for each condition and they showed the same trend.

**DLVO Energy Profiles.** The total interaction energy between \( E. coli \) and a flat collector surface was calculated using the Hogg et al. (1966) expression. Electrostatic
interaction ($\Phi_E$) was calculated based on surface potentials from section 2.4. The van der Waals attractive interaction energy was calculated using the Gregory (1981) approximation. A Hamaker constant between *E. coli* and each surface is presented in Table 1.

**SEM Sample Preparation.** All biofilm samples were fixed for SEM analysis using a method described previously (Clark et al. 2007). After fixation, biofilms were dried with a CO$_2$ critical point dryer (Tousimis, MD) and were sputter coated with gold-palladium. Biofilm samples were then viewed with a Philips XL30 field emission environmental scanning electron microscope (FEI, OR).

**Collection of OCT Biofilm Images.** OCT images of biofilm structures were captured in collaboration with the Biophotonics Imaging Laboratory at the Beckman Institute for Advanced Science and Technology (Nguyen et al. 2010, Xi et al. 2006). The Spectral-Domain OCT system for these studies utilized a mode-locked titanium:sapphire laser source (Kapteyn-Murnane Laboratories, Inc, Boulder, CO) centered at 800 nm with a bandwidth of 120 nm, providing an axial imaging resolution of 1.8 µm in water. The transverse resolution was 16 µm. The focus was set to be several centimeters beneath the glass surface of the sample holder where the biofilm structures were maintained. Two-dimensional cross-sectional images of 1 mm x 2 mm were acquired at an axial scan rate of 25 kHz, or at an approximate 40 ms acquisition time. The OCT system and OCT images are presented in the Supplemental Information.
Image analysis software developed under Matlab® (MathWorks, Natick, US) was used to analyze OCT images (Derlon et al. 2012). Image analysis consisted of the following steps:

1. detecting the membrane-biofilm interface (grey-scale gradient analysis);
2. binarizing the image (automatic thresholding);
3. calculating physical properties of the biofilm: mean biofilm thickness ($\bar{z}$ in µm), absolute ($R_a$ in µm) and relative roughness ($R_a'$) coefficients.

These parameters were calculated according to the following equations:

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

\[
R_a = \frac{1}{n} \sum_{i=1}^{N} \left( z_i - \bar{z} \right)
\]

\[
R_a' = \frac{1}{n} \sum_{i=1}^{N} \left( \frac{z_i - \bar{z}}{-\bar{z}} \right)
\]

where $N$ is the number of thickness measurements, $z_i$ is the local biofilm thickness (µm), and $\bar{z}$ is the mean biofilm thickness (µm).

**T-RFLP Analysis of Biofilms.** Biofilms grown from 2 to 25 weeks were collected for T-RFLP analysis (Liu et al. 1997). To collect the sample, PVC coupons were physically scraped with sterile cotton swabs. The cotton swabs were vortexed 3 times with the same buffer to retrieve the biomass as much as possible. The biomass-containing buffer solution was centrifuged at 12000×g and the pellets were kept at -80°C.
°C before DNA extraction. Biofilm community DNA was extracted according to a protocol developed for drinking water biofilms (Hwang et al. 2012). The extracted DNA was air dried and re-dissolved in 50 μL milli-Q water. The amount of extracted DNA was measured with Nanodrop (Thermo Scientific, DE) and stored at -80 °C for further PCR analysis. T-RFLP was conducted as described previously (Liu et al., 1997) using a primer set 47F and 927R targeting the domain Bacteria 16S rRNA gene. The forward primer was labeled with 6-FAM. PCR reactions were conducted in a Bio-rad 1000 thermal cycler (Bio-rad, CA). Each reaction product was examined by gel electrophoresis with 1% agarose gel in TAE buffer at 100V for 30 min. The final product was analyzed with ABI 3730 XL genetic analyzer at the Roy J. Carver Biotechnology Center at the University of Illinois. T-RFLP profiles were analyzed by Genemapper V 4.0. The peak binning was conducted with the Excel macro Treeflap (Rees et al. 2004). Statistical analysis was performed using PRIMER 6 software (Plymouth Marine Laboratory, UK). Relative abundance of terminal restrictive fragments (T-RFs) were tabulated, square-root transformed, and a distance matrix based on Bray-Curtis distance between samples was calculated. The similarities were visualized with cluster analysis.

2.4. Results and Discussion

Biofilm Imaging and Characterization. SEM and OCT imaging techniques were selected to characterize the physical properties of the biofilms at microscopic and mesoscopic resolution, respectively. SEM images (Fig. 2.4) showed that after 2 weeks of feeding with treated groundwater, only a small fraction of the PVC coupons in the CDC reactor was covered by biofilm, and after 4 weeks the entire surface of the
coupons was covered with mainly extracellular polymeric substances (EPS). The 2-week old biofilm was too thin to allow quantitative analysis of roughness with OCT. For 4-week and up to 24-week old biofilms, the changes in the biofilm roughness (Fig. 2.5A) and mean thickness (Fig. 2.5B) were monitored. The biofilm roughness increased from 11.7±3.5 µm to 17±1.3 µm until week 16, and then decreased to 8±0.5 µm between week 16 and 24 (Fig. 2.5A). At week 16, the mean biofilm thickness increased to 45±4 µm. At week 24, a stable mean biofilm thickness of 44±1.5 µm was obtained (Fig. 2.5C). Based on the thickness, roughness, and coverage data (qualitatively) determined by OCT and SEM, the biofilms seemed to be physically stable after 16 weeks.
Figure 2.4. SEM images of (A) PVC, (B) 2-week biofilm, (C) 4-week biofilm, (D) 16-week biofilm, (E) 24-week biofilm, and (F) 27-week biofilm at magnification of 10,000X. All biofilm samples were fixed, dried, and coated with Au before imaging.
Figure 2.5. OCT biofilm characterization data calculated by using algorithm in Matlab. A) absolute biofilm roughness (μm), B) relative biofilm roughness coefficient, C) mean biofilm thickness (μm).
Biofilm Community Analysis. Biological stability of the groundwater biofilms was determined by analyzing microbial community diversity with T-RFLP for biofilms ranging from 2 weeks to 25 weeks. The resulting electrophoregrams indicated that there was a shift in the major terminal restriction fragments (T-RFs). For the 2-week old biofilms, the 106-bp T-RF was detected to account for 75.9% of the total peak area (Fig. 2.6). The abundance of the same fragment was reduced to 24.5% for the 6-week old biofilms (Fig. 2.7). For biofilm samples after eight weeks (Fig. 2.8 to 2.12), major T-RFs were shifted to 88 bp and 400 bp, suggesting that a microbial succession was taking place during the CDC operation. The change in the most abundant T-RF suggests a change in the dominant groups of microbial communities during the biofilm development. During the entire experiment, T-RFs with fragment lengths of 83 bp, 95 bp, and 363 bp were observed in all biofilm samples.

The T-RFLP fingerprinting profiles were used to calculate the similarity index between samples, and to construct cluster analysis and determine the relative similarities among the communities. The cluster analysis results (Fig. 2.13) showed two separate clusters for “young” biofilms between 2 weeks and 6 weeks, and “old” biofilms longer than 14 weeks. This observation agreed with the physical characterization results based on SEM and OCT measurements. Among all biofilm samples, the highest similarity was observed among samples taken at 18, 20, and 25 weeks, suggesting that a stable microbial community was formed in biofilms at the late phase of the experiment.
**Figure 2.6.** T-RFLP profiles analyzed by Genemapper V 4.0. for 2-week biofilm.

**Figure 2.7.** T-RFLP profiles analyzed by Genemapper V 4.0. for 6-week biofilm.
Figure 2.8. T-RFLP profiles analyzed by Genemapper V 4.0. for 8-week biofilm.

Figure 2.9. T-RFLP profiles analyzed by Genemapper V 4.0. for 14-week biofilm.
Figure 2.10. T-RFLP profiles analyzed by Genemapper V 4.0. for 18-week biofilm.

Figure 2.11. T-RFLP profiles analyzed by Genemapper V 4.0. for 20-week biofilm.
Figure 2.12. T-RFLP profiles analyzed by Genemapper V 4.0. for 24-week biofilm.

Figure 2.13. Cluster analysis of T-RFLP for biofilm community diversity.

For biofilm samples taken between weeks 2 and 8, increasing thickness and roughness were observed together with a change in the microbial community structure.
of those young biofilms. This observation was in agreement with the incomplete coverage of the PVC coupons by 2-week old biofilm shown in the SEM image (Fig. 2.4). For 8-week and 14/16-week old biofilms, increasing thickness and decreasing roughness were correlated with unstable community structure as shown by cluster analysis. The observation that the biofilm community became stabilized between weeks 18 and 25 was consistent with similar thickness and roughness observed for 16-week and 24-week old biofilms. The SEM images for 16-, 24-, and 27-week old biofilms also showed that the PVC coupons were completely covered with biofilms. In summary, both physical and biological biofilm characteristics suggest that the development of biofilms was mostly taking place from week zero to 16 weeks, and reached stable biofilm structure after 18 weeks of feeding. The general classification between young and old biofilms was further used to explain deposition of *E. coli* cells and CML particles on biofilms.

**Electrophoretic Mobility.** The physical interactions between colloid particles and another surface have been traditionally described by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Elimelech et al. 1995). According to this theory, the surface charge and Hamaker's constant are the two main parameters that are used to determine the electrostatic and van der Waals interactions. The surface charge of *E. coli* cells, CML particles, and biofilms was assumed to be equal to their zeta potential. Electrophoretic mobilities were measured and converted to zeta potential using the Smoluchowski equation (Elimelech et al. 1995). Figure 2.14 shows the electrophoretic mobility of biofilms obtained at different growth periods. Less negative electrophoretic mobility was observed with increasing ionic strength. However, at a given ionic strength,
comparable values for electrophoretic mobility of biofilms taken at weeks 2, 6, and 8 were observed. Specifically, electrophoretic mobility at 3 mM KCl was \(-1.5\pm0.08\) and \(-1.4\pm0.1\) μmVs\(^{-1}\)cm\(^{-1}\) for 2- and 6-week old biofilms, respectively. At 10 mM ionic strength, the 4- and 27-week old biofilms showed slightly more negatively charged electrophoretic mobility than the 2-, 6-, and 8-week old biofilms. However, at 300 mM, all biofilms had statistically similar electrophoretic mobility. For 6-week old biofilms, electrophoretic mobilities measured in 10 mM and 300 mM ionic strength solutions were similar for samples sonicated for 5 min or 30 min. Specifically, electrophoretic mobilities of 6-week biofilms were \(-1.4\pm0.14\) and \(-1.5\pm0.15\) μmVS\(^{-1}\)cm\(^{-1}\) for 5 and 30 min sonication. Apparently, the duration of sonication did not have influence on the EM result, as it showed no difference in zeta potential at different sonication times. However, sonication of biofilms was likely to remove the entire biofilm from the PVC coupons, and the electrophoretic measurement represented the whole biofilm, not just the biofilm surface. The electrophoretic mobility and the corresponding zeta potential of \textit{E. coli} cells in the presence of KCl are presented in Figure 2.14. All measurements were measured at 25 °C and at pH 8.2-8.5 for ionic strengths ranging from 3 to 300 mM KCl.
Figure 2.14. Electric surface charge properties of *E. coli* S17 (square), 2-week biofilm (triangle), 4-week biofilm (open star), 6-week biofilm with 5-min sonication (open circle), 6-week biofilm with 30-min sonication (closed star), 8-week biofilm (diamond), 27-week biofilm (cross) as a function of ionic strength (KCl) at pH 8.2-8.5. Zeta potential was calculated from experimental electrophoretic mobility using Smoluchowski equation.

Under all experimental conditions, the electrophoretic mobility of *E. coli* cells were negative, and became less negative with increasing salt concentrations. The EM of *E. coli* cells were -3.3 μmVs⁻¹cm⁻¹ and -0.5 μmVs⁻¹cm⁻¹ at 3 mM and 600 mM, respectively. The observed EM for *E. coli* cells were more negative than those of other *E. coli* strains reported by Walker et al. (2006) because of higher pH (8.2-8.5) used in this current work. The electrophoretic mobility of *E. coli* has been reported to become more negative with increasing pH (Kim et al. 2009). Compared to *E. coli* cells, CML particles were more negatively charged (Fig. 2.14). Higher electrostatic repulsion was
expected for CML deposition on biofilm compared to *E. coli* deposition on the same biofilm.

**Contact Angle of *E. coli* cells and Other Substrates.** The van der Waal interaction between the biofilms and *E. coli* cells or CML particles is determined by the Hamaker’s constant (A). The constants for *E. coli*-biofilm-water and CML-biofilm-water were calculated from the contact angles of diiodomethane on *E. coli* cells, CML, PVC, and biofilms at different ages. As shown in Table 1, the contact angle of non-polar hydrophobic diiodomethane on *E. coli* cells (70.6°±2.2°) was the most polar and hydrophilic followed by CML (55.1°±2°), PVC (49.8°±2.2°), and biofilms (26° to 43°). With increasing biofilm age, the contact angle became smaller, which suggested a less polar surface and more hydrophobic biofilm surface.
Table 2.1. Contact angle and corresponding Hamaker’s constant (A) of biofilms, PVC, CML, and *E. coli* using diiodomethane as a liquid probe. Contact angles were measured by sessile drop using goniometer. Hamaker’s constant was calculated from contact angle.

<table>
<thead>
<tr>
<th></th>
<th>(\Theta_{\text{diiodomethane}})</th>
<th>(\gamma_{\text{LW}}) (mJ/m(^2))</th>
<th>(\Delta G) (mJ/m(^2))</th>
<th>A (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC</td>
<td>49.8 ± 2.2</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm 2wk</td>
<td>43.2 ± 1.5</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm 4wk</td>
<td>34.5 ± 1.3</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm 6wk</td>
<td>36.3 ± 2.3</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm 8wk</td>
<td>35.0 ± 4.7</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm 16wk</td>
<td>33.7 ± 2.9</td>
<td>42.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm 24wk</td>
<td>26.6 ± 2.9</td>
<td>45.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm 27wk</td>
<td>26 ± 1.0</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> S17</td>
<td>70.6 ± 2.2</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CML</td>
<td>55.1 ± 2</td>
<td>31.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>(\gamma_{\text{LW}}) (mJ/m(^2))</th>
<th>(\Delta G) (mJ/m(^2))</th>
<th>A (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML-water-PVC</td>
<td>-6.6×10(^{-4})</td>
<td>6.1×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td>CML - water - BF 2 wk</td>
<td>-5.0×10(^{-4})</td>
<td>4.6×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td>CML - water - BF 4 wk</td>
<td>-3.4×10(^{-4})</td>
<td>3.2×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td>CML - water - BF 8 wk</td>
<td>-3.4×10(^{-4})</td>
<td>3.2×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td>CML - water - BF 16 wk</td>
<td>-3.2×10(^{-4})</td>
<td>3.0×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td>CML - water - BF 24 wk</td>
<td>-2.0×10(^{-4})</td>
<td>1.9×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td>CML - water - BF 27 wk</td>
<td>-2.3×10(^{-4})</td>
<td>2.1×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> - water - PVC</td>
<td>-1.6×10(^{-3})</td>
<td>1.5×10(^{-21})</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> - water - BF 2 wk</td>
<td>-1.3×10(^{-3})</td>
<td>1.2×10(^{-21})</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> - water - BF 4 wk</td>
<td>-1.1×10(^{-3})</td>
<td>9.8×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> - water - BF 6 wk</td>
<td>-1.1×10(^{-3})</td>
<td>1.0×10(^{-21})</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> - water - BF 8 wk</td>
<td>-1.1×10(^{-3})</td>
<td>9.8×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> - water - BF 16 wk</td>
<td>-1.1×10(^{-3})</td>
<td>9.8×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> - water - BF 24 wk</td>
<td>-1.1×10(^{-3})</td>
<td>9.8×10(^{-22})</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> - water - BF 27 wk</td>
<td>-8.4×10(^{-4})</td>
<td>7.8×10(^{-22})</td>
<td></td>
</tr>
</tbody>
</table>

Lifshitz-van der Waals components of free energy of adhesion (\(\Delta G_{y0}^{\text{LW}}\)) between *E. coli* and CML and the collector surfaces are listed in Table 2.1. \(\Delta G_{y0}^{\text{LW}}\) was less
negative with increasing biofilm ages. As a result of less negative values of $\Delta G_{LW}^{i_0}$ for older biofilms, the Hamaker’s constant ($A$) was calculated based on Lifshitz-van der Waals components of free energy of adhesion ($\Delta G_{LW}^{i_0}$) between $E. coli$ and CML and each surface became smaller (Table 2.1). The Hamaker’s constant of $E. coli$ - water - biofilm ($1.5 \times 10^{-21}$ J) measured here is comparable to the Hamaker’s constant of $E. coli$ - water - quartz used in literatures ($6.5 \times 10^{-21}$ J) (Redman et al. 2004, Walker et al. 2006, Walker et al. 2004), and cryptosporidium oocyst – water – quartz ($6.5 \times 10^{-21}$ J) (Liu et al. 2010).

**Total Energy Barrier Between $E. coli$ and a Biofilm.** The zeta potential and Hamaker constants determined above were used to calculate the energy barrier between biofilms and $E. coli$ cells or CML particles according to the DLVO theory (Table 1.2). As shown above, similar values of zeta potential for biofilms grown at different lengths of time (Fig. 2.14) suggested that the repulsive interaction between $E. coli$ cells and the biofilms is similar for these biofilms. The values of Hamaker’s constant ($A$) determined from the contact angle measurement were used to calculate the van der Waals energy interaction components for the DLVO energy profiles. Smaller Hamaker’s constants (Table 2.1) indicated weaker van der Waals interaction for older biofilms. However, the small difference in van der Waals interaction was overwhelmed by repulsion interaction. As a result, energy barriers were present for 3, 10, and 70 mM (Table 2.2). For example, interaction energies of 2- and 8-week old biofilms in 10 mM were 308 and 313 kT, respectively. For 27-week old biofilms in 10 mM, the interaction energy was 601 kT, which was higher than interaction energies of 2- and 8-week old biofilms due to the more negative electrophoretic mobility. Interaction energies between
CML and different ages of biofilms at 10 mM KCl also showed the same trend as \textit{E. coli} cells. For example, the interaction energies of 2- and 8-week old biofilms in 10 mM were 357 and 367 kT, respectively. For 27-week old biofilms in 10 mM, the interaction energy was 798 kT. These high interaction energies suggest low or no deposition of \textit{E. coli} cells or CML particles on biofilms. Moreover, the fact that interaction energies are present at every ionic strength suggested that low deposition rates of \textit{E. coli} cells or CML particles would be observed.

\textbf{Table 2.2.} A) Interaction energy between \textit{E. coli} and biofilms at different age and ionic strength, and B) Interaction energy between CML and biofilms at different age and ionic strength.

\begin{table}[h]
\begin{center}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{IS (mM)} & \textbf{2 week} & \textbf{6 week} & \textbf{8 week} & \textbf{27 week} \\
\hline
3 & 342 & 315 & 463 & - \\
10 & 308 & 284 & 313 & 601 \\
70 & 75 & 45 & 50 & - \\
300 & 0 & 0 & 0 & 0.5 \\
\hline
\end{tabular}
\end{center}
\end{table}

\begin{table}[h]
\begin{center}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{IS (mM)} & \textbf{2 week} & \textbf{6 week} & \textbf{8 week} & \textbf{27 week} \\
\hline
3 & 374 & 345 & 523 & - \\
10 & 357 & 326 & 367 & 798 \\
70 & 171 & 112 & 120 & - \\
300 & 105 & 65 & 93 & 179 \\
\hline
\end{tabular}
\end{center}
\end{table}

As predicted by the DLVO theory, energy barriers decreased with ionic strength. Specifically, on 2-week old biofilms, interaction energies at 3 and 300 mM were 341.7 and 0 kT, respectively. On 27-week old biofilm, interaction energies at 10 and 300 mM,
which were 601.2 and 0.5 kT, respectively, decreased with ionic strength. However, the presence of energy barriers were observed even at high ionic strength, suggesting that deposition of *E. coli* cells on biofilms should be unfavorable.

**Deposition Kinetics of *E. coli* Cells and CML Particles.** The deposition kinetics data for *E. coli* cells and CML particles on biofilms and PVC coupons were obtained and compared with the trends predicted by the DLVO theory. Deposition rate coefficients of *E. coli* cells on PVC surface and 2-week old biofilm were observed to increase with ionic strength (Fig. 2.15). Specifically, $k_D$ of *E. coli* cells on PVC increased from $(7.9\pm1.3)\times10^{-9}$ to $(3.4\pm0.8)\times10^{-7}$ m/s at 3 and 300 mM, respectively. The same trend was observed for CML particles. The $k_D$ of CML on PVC increased from $(4.5\pm0.6)\times10^{-8}$ to $(5.9\pm0.3)\times10^{-7}$ m/s at 3 and 300 mM, respectively. On 2-week old biofilms, $k_D$ of *E. coli* cells and CML particles showed the increasing trend from low to high ionic strength as well. As observed by SEM, 2-week old biofilms were not yet fully established on PVC surface, and *E. coli* cells or CML particles were likely to deposit on both the biofilm surface and uncovered PVC surface. The increase in deposition rates with solution ionic strength could be expected based on DLVO theory because less negative surface charge of *E. coli* cells, CML, and biofilm in higher ionic strength solutions due to the compression of double layer thickness could lead to higher deposition. Thus, for PVC and 2-week old biofilms, electrostatic interaction played an important role in controlling both *E. coli* cells and CML particle deposition.
The deposition rate coefficients of *E. coli* cells measured at ionic strengths from 3 mM to 300 mM were statistically the same (*p* < 0.05) on biofilm from week 6 and older. Specifically, *k*D of *E. coli* cells on 6-week old biofilm were (1.3±0.3)×10⁻⁷ and (1±0.1)×10⁻⁷ m/s at 3 and 300 mM, respectively. On the biofilms at 27 weeks, the deposition rate coefficients of *E. coli* cells were (1.0±0.1)×10⁻⁷ and (1.4±0.4)×10⁻⁷ m/s at 10 and 300 mM, respectively. Deposition rate coefficients of CML particles on 8-week old biofilms were also found to be independent of ionic strength. Specifically, *k*D of CML on 8-week old
biofilms were $(1.3\pm0.4)\times10^{-6}$ and $(1.2\pm0.4)\times10^{-6}$ m/s at 3 and 300 mM, respectively. The observation that deposition was independent of ionic strength for both E. coli cells and CML particles qualitatively disagreed with lower energy barriers calculated from DLVO theory for these biofilms (Table 1.2). It is likely that the deposition of E. coli cells or CML particles on 8-week and older biofilms was not mainly controlled by classic DLVO forces such as electrostatic and van der Waals interactions. The surface structure of biofilms such as thickness and roughness was further investigated.

Deposition rate coefficients of biofilms were found to increase with biofilm relative roughness (Fig. 2.16). Specifically, deposition rate coefficients of E. coli cells increased from $(1.0\pm0.1)\times10^{-7}$ to $(1.9\pm0.2)\times10^{-7}$ m/s for 16 and 8 week-biofilms, while biofilm roughness coefficient changes from $0.2\pm0.03$ to $0.55\pm0.04$. In addition, deposition rate coefficients of E. coli cells on biofilms at weeks 16 and 24 were similar ($(1.0\pm0.1)\times10^{-7}$ m/s and $(1.3\pm0.3)\times10^{-7}$ m/s, respectively), and so were the biofilm surface roughness coefficients ($0.2\pm0.03$ to $0.2\pm0.01$, respectively). The physical biofilm structure in terms of relative roughness also influenced the CML deposition (Fig. 2.16). Deposition rate coefficients of CML increased from $(1.1\pm0.07)\times10^{-6}$ m/s to $(2.4\pm0.2)\times10^{-6}$ m/s for 16 and 8 week-biofilms, respectively. The deposition rate coefficients of CML were also similar for 16 and 24-week biofilms ($(1.1\pm0.07)\times10^{-6}$ m/s and $(1.0\pm0.02)\times10^{-6}$ m/s, respectively).
Figure 2.16. Deposition rate coefficient \( k_D \) of *E. coli* S17 as a function of biofilm relative roughness coefficient at different biofilm ages. Deposition experiments were carried out at 10 mM KCl, pH 8.2-8.5 at room temperature. For 2-week biofilm, the biofilm roughness coefficients were undetected because the biofilms were too thin for OCT imaging.

Our observation that both *E. coli* and CML particles had higher deposition on biofilms with higher roughness is consistent with previous works on particle deposition on glass, metal and membrane surfaces (Chen et al. 2010, Diaz et al. 2007, Shellenberger and Logan 2002, Subramani and Hoek 2010). For example, enhanced *E. coli* cell and latex bead deposition on rough glass beads was higher than those on smooth glass beads (Shellenberger and Logan 2002). Moreover, Diaz et al., (2007) found that the bacterial arrangement on metal surfaces was influenced by a structure of the substrate itself. More than 76% of isolated cells attached to the rough surface, fitted into the trenches and aligned with the trenches. Higher deposition of latex particles on metal surfaces was increased with surface roughness (Chen et al. 2010). An extended DLVO theory has been developed to explain enhanced attachment of particles on rough surfaces. According to this model, for like-charged surfaces, the energy barrier
estimated between the colloids and the polymeric membrane surface with semispherical asperities was lower compared to that between the colloids and the smooth membrane surface to allow more deposition on rough surface compared to deposition on smooth surface (Hoek and Agarwal 2006, Hoek et al. 2003, Huang et al. 2010).

As discussed above, lower Hamaker constant and more negative zeta potential for CML compared to *E. coli* cells suggested that the energy barrier between CML and the glass surface should be higher than that between *E. coli* cells and the glass surface (747 kT for CML vs. 580 kT for *E. coli*). Higher energy barrier should lead to lower deposition. In contrast to our expectation, the deposition of CML is 10 time higher than *E. coli* at all biofilm ages and ionic strengths (Fig. 2.16). Specifically, $k_D$ of CML and *E. coli* on 2-week biofilm is $(1.8\pm0.1)\times10^{-7}$ and $(1.6\pm0.2)\times10^{-8}$, respectively. In addition to deposition on PVC coupons, an additional set of *E. coli* cells and CML deposition on clean glass surface in 10mM ionic strength solution was conducted. A similar trend for the 2-week biofilm was observed; $k_D$ of CML and *E. coli* on the glass surface was $(1.6\pm0.2)\times10^{-6}$ and $(4.8\pm0.7)\times10^{-9}$. In addition to DLVO interactions that control *E. coli* deposition on the glass surface, steric repulsion due to the presence of macromolecules on the *E. coli* cell surface may lower *E. coli* deposition compared to the deposition of CML particles. This steric interaction has previously been found for bacteria and oocyst deposition (Liu et al. 2010, Rijnaarts et al. 1999).

To test the effect of water hardness on *E. coli* cell deposition on biofilms, the deposition experiment of *E. coli* in Newmark groundwater was conducted on the 24-week biofilms in the presence of filtered groundwater. The deposition rate coefficients were statistically the same ($p < 0.05$) for groundwater and solutions containing 3 or 10
mM KCl \((1.2\pm0.2)\times10^{-7}\) for 3mM, \((1.3\pm0.3)\times10^{-7}\) for 10 mM, and \((1.6\pm0.6)\times10^{-7}\) m/s for groundwater). Newmark groundwater (pH 7.8) had an alkalinity of 330 mg/L as CaCO\(_3\), and contained 1.5 mM Ca\(^{2+}\) and 1.0 mM Mg\(^{2+}\). The similarity of \textit{E. coli} deposition in solution with and without hardness suggested that typical hardness of drinking water did not influence \textit{E. coli} deposition.

2.5. \textit{Conclusions}

- The mechanisms of \textit{E. coli} attachment change depending on the age of the biofilms.
- The physico-chemical properties of the water (ionic strength, hardness) govern the deposition rate of \textit{E. coli} cells and CML particles on PVC surfaces and on young/thin biofilms (age < 8 weeks). An increasing ionic strength/hardness increases the deposition rates.
- The physical biofilm properties govern \textit{E. coli} cell attachment in the case of mature biofilms (age > 16 weeks). An increasing biofilm roughness increases the deposition rate.

2.6. \textit{Acknowledgement}

This publication was made possible by USEPA grant R834870. Its contents are solely the responsibility of the grantee and do not necessarily represent the official views of the USEPA. Further, USEPA does not

2.7. \textit{References}


mycobacteria in drinking water distribution systems. Applied and Environmental Microbiology 67(3), 1225-1231.


CHAPTER 3

ROLE OF DISINFECTANTS ON ADHESION KINETICS OF *ESCHERICHIA COLI* AND *LEGIONELLA PNEUMOPHILA* ONTO GROUNDWATER AND TAP WATER BIOFILM GROWN ON PVC SURFACES

3.1 *Research Summary*

<table>
<thead>
<tr>
<th>Research Questions</th>
<th>Experiments conducted</th>
<th>Summary of results</th>
</tr>
</thead>
<tbody>
<tr>
<td>How does disinfectant influence biofilm properties?</td>
<td>1) EPM of biofilms</td>
<td>1) The NH$_2$Cl treated biofilms had more negative EPM compared to those not exposed to NH$_2$Cl. However, biofilm grown from deCl$_2$ have the same EPM with and without exposure to tap water.</td>
</tr>
<tr>
<td></td>
<td>2) Hydrophobicity of biofilms</td>
<td>2) Hydrophobicity remained the same for 20wk and 12wk NH$_2$Cl treated GW biofilms.</td>
</tr>
<tr>
<td></td>
<td>3) Biofilm surface structure</td>
<td>3) Hydrophobicity also remained the same for biofilm grown from deCl$_2$ with or without exposure to tap water.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4) After NH$_2$Cl treatment, thickness remained the same, but roughness coefficient decreased.</td>
</tr>
<tr>
<td>Does ionic strength control adhesion of bacterial cells on biofilms with and without being exposed to disinfectants?</td>
<td>4) Adhesion of <em>E. coli</em> 14f cells on PVC and young biofilms as a function of IS (3 to 300 mM KCl).</td>
<td>5) Deposition on old biofilms before and after NH$_2$Cl treatment didn’t depend on IS. However, a different trend was observed for tap water treatment.</td>
</tr>
<tr>
<td></td>
<td>5) Adhesion of <em>E. coli</em> 14f cells on mature biofilms (8 weeks and more) as a function of IS (3 to 300 mM KCl).</td>
<td></td>
</tr>
<tr>
<td>Does bacterial surface hydrophobicity have an influence on bacterial adhesion on a surface?</td>
<td>6) Adhesion of 3 different strains (<em>E. coli</em> 14f, <em>E. coli</em> S17, and <em>Legionella</em> on a clean PVC surface)</td>
<td>6) On PVC adhesion of <em>Legionella</em> and S17 was greater than 14f due to affinity of cell-surface hydrophobicity.</td>
</tr>
<tr>
<td></td>
<td>7) Adhesion on 11-week and</td>
<td>7) Hydrophilic <em>E. coli</em> 14f</td>
</tr>
</tbody>
</table>
20-week biofilms deposit more on biofilms than hydrophobic *E. coli* S17 and *Legionella*.

| Does biofilm roughness control the adhesion of bacterial cells onto the biofilms? | 8) Grown biofilms in groundwater at different age. 9) Introduce two types of disinfectants to treat biofilms.  - Monochloramine (NH₂Cl) to treat GW biofilms.  - Tap water (free Cl₂) to treat biofilms grown with dechlorinated tap water | 8) Adhesion of all strains depended on biofilm roughness. 9) After NH₂Cl treatment, roughness of biofilm decreased as well as adhesion. |

### 3.2. Abstract

Adhesion kinetics of two *E. coli* strains and a *Legionella pneumophila* strain on biofilms were studied using a parallel flow plate chamber (PFPC) coupled with confocal laser scanning microscope (CLSM) and epifluorescence microscope. Biofilms were grown with groundwater and dechlorinated tap water on PVC coupon in CDC reactors, and were subsequently disinfected with monochloramine and chlorine for 3 months. The biofilm structure was determined with optical coherent tomography (OCT) or CLSM, depending on the biofilm thickness. OCT results revealed that 12 weeks of exposure to monochloramine led to smoother but not thinner biofilms. For all three studied strains, adhesion kinetics presented as Sherwood numbers were linearly correlated with the biofilm roughness coefficients. *E. coli* and *Legionella* adhesion on groundwater biofilms was lowered after biofilms were exposed to monochloramine, consistent with reduced biofilm roughness from exposure. Twelve-week exposure to tap water of biofilm grown
from dechlorinated tap water for 8 weeks also led to lower adhesion of environmental *E. coli*. The results from this study showed that disinfectants did not completely eradicate biomass but rather changed their roughness, which subsequently led to lower attachment of bacteria.

### 3.3. Introduction

Despite advances in water treatment technologies, drinking waterborne outbreaks continue to occur in the United States. In 2009-2010, 33 drinking water-associated outbreaks were reported, resulting in 1,040 cases of illness, 85 hospitalizations, and 9 deaths (Hilborn et al., 2013). Causes of the reported outbreaks include post-contamination of drinking water after treatment. Contamination of drinking water occurs in drinking water distribution systems (DWDS) due to factors such as distribution system age, pipe integrity, cross-connection and low pressure (Snoeyink et al., 2006). Premise plumbing system, a portion of the potable water distribution system associated with hospitals, houses, schools, and other buildings are more susceptible to biological contaminations than the main service line due to its specific characteristics. Premise plumbing may have low concentration of residual disinfectants, frequent and variable stagnation of the water, and extreme temperatures (Snoeyink et al., 2006). Furthermore, the integrity of the water in premise plumbing is not regulated by the U.S. Environmental Protection Agency (Fraser et al., 1977). The Safe Drinking Water Act requires only sample collection for lead and copper but not for disinfectant residuals at the tap after the water has been left stagnant (Snoeyink et al., 2006). With limited control, premise plumbing tends to facilitate microorganism regrowth and biofilm formation, which can lead to further, more complex problems such as biofilms protecting
and harboring pathogens from disinfectants in DWDS (Kim et al., 2002; Szewzyk et al., 2000).

Appropriate residual disinfectant concentration in the main distribution pipeline is required by the U.S. EPA to disinfect planktonic pathogens and to control biofilm growth. Many previous studies have shown that chlorine and monochloramine are effective in inactivation of planktonic microorganism. For example, *Legionella pneumophila* and *Escherichia coli* are 99% inactivated within 40 min and 1 min, respectively, at free chlorine concentration of 0.1 mg/L (Kuchta et al., 1983; Yabuchi et al., 1995). However, the contact time and concentration of disinfectants have to be significantly increased to achieve the same disinfection results when biofilms are present. *Legionella* cultures associated with biofilms are 100 times more resistant to disinfectants than those floating in water (Cargill et al., 1992; Muraca et al., 1987; Yaradou et al., 2007).

Thorough studies have been conducted to show how diffusional effects decrease disinfectant effectiveness within biofilm matrices. Disinfectants have been shown to react with extracellular polymeric substances (EPS) of the biofilm matrix before diffusing into the biofilm (Chen and Stewart, 1996; Gagnon et al., 2005; Lee et al., 2011; Ntsama et al., 1997; Xue et al., 2012). The diffusivity of chlorine in pure artificial agarose is 2-3 times higher than in agarose associated with *P. aeruginosa* ERC 1 and biofilms produced from these cells; this demonstrates that the reaction of chlorine with cellular biomass is significant enough to limit the diffusion rate of chlorine into the agarose matrix (Chen and Stewart, 1996). A further study of *P. aeruginosa* biofilms also shows that biofilms producing more EPS retain more cell viability in the core of the biofilm.
matrix after chlorine disinfection, than non-EPS-producing biofilms which were almost fully disinfected throughout (Xue et al., 2012).

However, it is not clear how the biofilm physical structure affect the transfer of disinfectants and the activation of pathogenic bacteria. Although the viability of bacterial cells within biofilm matrices is decreased by the effect of disinfectants, physical biofilm structures may not be altered by those disinfectants. Biofilm structures have been shown to influence bacterial adhesion (Hilbert et al., 2003; McAllister et al., 1993; Subramani et al., 2009) but these studies fall short of mentioning the effect of disinfectant on the biofilm structures and subsequent bacterial attachment. Another study has shown that chlorine can interrupt hydrogen bonding, polymeric interactions, and hydrophobic interactions, leading to the detachment of single cells and certain parts of biofilms, but the same detached clusters were able to survive in the presence of chlorine and later-on reattach themselves to the larger biofilm surface (Xue and Seo, 2013). Biofilms were found to become thinner and more compacted from exposure to monochloramine (Ling and Liu, 2013). However, the question on whether this structural change affected bacterial adhesion onto biofilms was not answered.

Our study aims to elucidate the mechanisms that influence bacterial adhesion on disinfected biofilms. Three different strains of bacteria; *Legionella pneumophila*, environmental *E. coli* 14f, and laboratory strain *E. coli* S17, were used in the experiment. Biofilms were grown on PVC coupons with four different types of water: groundwater, chloraminated groundwater, dechlorinated tap water, and tap water. The adhesion of three different strains of bacteria on biofilms was observed using an inverted fluorescence microscope coupled with a parallel flow plate chamber (PFPC) at
ionic strength ranging from 3 to 300 mM KCl at pH 8.2. Thickness and roughness of biofilms grown under different conditions (e.g. under influence of chlorine and monochloramine) were measured by optical coherence tomography (OCT) and confocal laser scanning microscopy (CLSM).

3.4. Materials and Methods

Biofilm Growth in CDC Reactor. Biofilms on PVC coupons were grown in a Center of Disease Control (CDC) reactor according to previously described protocol (Janjaroen et al., 2013). All reactors were operated at 125 rpm and at a hydraulic retention time (HRT) of 1.4 hr. Biofilms were grown for 8 weeks using groundwater or dechlorinated tap water (3 reactors for each type of water; see Figure 3.1-3.2). To remove chlorine from tap water, sodium thiosulfate at a concentration of 0.9 mM was used to quench chlorine daily. Total and free chlorine (Cl₂) concentrations in untreated and treated tap water were measured with a DPD colorimetric method (Hach Method 8167 for total Cl₂ and Hach Method 8021 for free Cl₂).
Figure 3.1. Three lines of groundwater biofilm reactors. The 1\textsuperscript{st} and 3\textsuperscript{rd} lines were a control; biofilms were grown on PVC coupons with groundwater for 8 and 20 weeks. The reactor in the 2\textsuperscript{nd} line was introduced to monochloramine disinfection for 12 weeks. At the end of each period, biofilms in each line were taken out and used for surface characterization and adhesion experiments.

Figure 3.2. Three lines of dechlorinated tap water biofilm reactors. The 1\textsuperscript{st} and 3\textsuperscript{rd} lines were a control; biofilms were grown on PVC coupons with dechlorinated tap water for 8 and 20 weeks. The reactor in the 2\textsuperscript{nd} line was introduced to chlorine disinfection for 12 weeks. At the end of each period, biofilms in each line were taken out and used for future surface characterization and adhesion experiments.
**Disinfection Experiment of Biofilm.** Disinfection experiments began at the end of the 8-week biofilm growth period. Of the three CDC reactors developed with each different kind of water (groundwater, and dechlorinated tap water), biofilm from one reactor was set aside for further experiments, another was disinfected for 12 weeks, and the third was kept as control and not disinfected but was continually fed with either groundwater or dechlorinated tap water for another 12 weeks (Figure 3.1-3.2). Laboratory-prepared monochloramine was added to the reactor fed with groundwater.

Monochloramine disinfectant solution was prepared daily by mixing sodium hypochlorite with ammonium chloride at a mass ratio (Cl₂ to NH₃-N) of 4:1. The solution was buffered with 0.5M sodium bicarbonate (pH 8.2). The final total and free Cl₂ concentration in the reactor was maintained between 2-4 mg/L and 0.02 mg/L as Cl₂ to represent the requirements outlined by Illinois State Law. Tap water containing free chlorine replaced treated tap water as feed for the CDC reactor with biofilm developed from treated tap water intended for disinfection. By the 20th-week since initiation of biofilm development, biofilms from all CDC reactors were taken out for adhesion experiments and surface structure analysis.

**Biofilm and PVC Imaging and Analysis.** Due to the different thickness of biofilms grown from groundwater against biofilms grown from tap water, different imaging techniques had to be employed. The thicker groundwater biofilms were imaged and analyzed with Optical Coherence Tomography (OCT), which is only suitable for analyzing biofilms that exceed 2 µm in thickness (minimum resolution). Biofilms were placed in a PFPC in groundwater. Images were taken with the Spectral-Domain OCT system utilizing a mode-locked titanium:sapphire laser source (Kapteyn-Murnane...
Laboratories, Inc, Boulder, CO) centered at 800 nm with a bandwidth of 120 nm, providing an axial imaging resolution of 1.8 µm in water. Transverse resolution was 16 µm with the focus set several centimeters beneath the glass surface close to the biofilm structures. Cross-sectional images were acquired at 1 mm × 2 mm by an axial scan rate of 25 kHz. Image analysis program (Derlon et al. 2012) was used to process and analyze biofilm images for roughness and thickness. Biofilm refractive index (RI) was determined experimentally to be approximately 1.4±0.15. RI is used to normalize average thickness measured by OCT to correctly achieve true biofilm thickness. Calculation details can be found below.

The same version of the image analysis program that was used in Derlon et al. (2012) was used to analyze the OCT images. Image analysis consisted of the following steps:

1. Detecting the membrane-biofilm interface (grey-scale gradient analysis);

2. Binarizing the image (automatic thresholding);

3. Calculating the physical properties of the biofilm: mean biofilm thickness (∼ in µm), absolute (Rₐ in µm) and relative roughness (Rₐ') coefficients.

These parameters were calculated according to the following equations:

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

(1)

\[
R_a = \frac{1}{n} \sum_{i=1}^{N} \left( |z_i - \bar{z}| \right)
\]

(2)
\[
R'_u = \frac{1}{n} \sum_{i=1}^{N} \left( \frac{z_i - \bar{z}}{\bar{z}} \right)
\]

(3)

Where \( N \) is the number of thickness measurements, \( z_i \) is the local biofilm thickness (\( \mu m \)), and \( \bar{z} \) is the mean biofilm thickness (\( \mu m \)).

Thinner tap water biofilms were imaged and analyzed with CLSM technique, suitable for thin biofilm analysis due to the characteristic of the dye's limited penetration. LIVE/DEAD® BacLight™ stain (L7012; Invitrogen; Calsbad, California) was used to dye the bacterial cells inside biofilm matrices. 300 \( \mu L \) of dye mixture was added to the surface of biofilms and allowed to incubate for 20 min at room temperature. Biofilms disinfected with untreated tap water had to be dechlorinated before staining with sodium hypochlorite to reduce the interference of chlorine over the dye signal. CLSM was used to capture images of biofilm matrix with red and green channel. Green signal was used in COMSTAT (Heydorn et al., 2000a, 2000b) algorithm to calculate biofilm roughness and thickness for nascent biofilms, while the ratio of green and red signals was used to calculate the live/dead ratio of disinfected biofilms. In order to receive an accurate measurement, the threshold of each image should be selected such that all bacterial cells can be detected.

Surface profiler (Dektak 3030; Veeco; Plainview, NY, USA) was used to measure clean PVC surface height and roughness. A diamond tipped stylus of 12.5 \( \mu m \) was a scanning probe at the scanning rate of 1 \( \mu m/s \). The vertical resolution at this scan rate was around 1 nm. Measurements were made by the sample stage moving underneath
the diamond stylus. Surface variation caused the stylus to be moved vertically. According to this movement, electrical signals are changed from analog to digital form using an integrating A-D converter. Sample surface height and roughness are calculated based on equations used in OCT analysis.

**Fluid Dynamics in a Parallel Flow Plate Chamber (PFPC).** Fluid dynamics and flow profile in the flow chamber was solved numerically with COMSOL; the Navier-Stokes equation for incompressible, laminar flow was selected for this study. The initial velocity was calculated from the flow rate and cross-sectional area of flow. The boundary layer for both biofilm and glass surface was defined as ‘no slip’. Fluid velocity profile over biofilms and PVC surfaces were acquired after computation. Reynolds number \(Re\) of the flow cell was calculated based on the characteristic length and flow rate of the flow cell (Bakker et al., 2003). Calculated \(Re\) \((Re = 1.24)\) was further used to check the validity of laminar flow assumption. Furthermore, the effect of flow rate on the change in biofilm roughness was also tested with higher flow rates. Shen et al. (publication in preparation) found that with rigid groundwater biofilms, higher flow rates did not significantly change biofilm structure determined by OCT.

Reynold number \(Re\) for the flow cell and a single bacteria cell was calculated using the following equations (Bakker et al., 2003; Clark, 2009).

\[
Re_{cell} = \frac{a_p \times V \times \rho}{\mu} \quad (4)
\]

\[
Re_{flowcell} = \frac{Q \times \rho}{(w + 2b) \times \mu} \quad (5)
\]
Where \( a_p \) is the spherical equivalent diameter of bacteria cells (m), \( V \) is the flow velocity in the flow cell (0.003 m/s in this study), \( \mu \) is the viscosity of the electrolyte solution used in this experiment (kg.m\(^{-1}\).s\(^{-1}\)), \( Q \) is the flow rate in m\(^3\)/s, \( w \) is the width of the flow cell chamber (m), and \( b \) is the half depth between a coupon surface and a glass cover slip (m). In our study, \( Re_{\text{Legionella}} \), \( Re_{\text{E. coli}} \) and \( Re_{\text{flowcell}} \) are 0.005, 0.007 and 1.24, respectively.

Peclet number (Adamczyk and Van De Ven, 1981) was also determined for bacterial cell mass transfer in the flow cell. \( Pe \) represents the ratio between convection mass transfer and diffusion mass transfer (Clark, 2009).

\[
Pe_{\text{cell}} = \frac{3V \times a_p^3}{2D \times b^2} \tag{6}
\]

Where \( D \) is the diffusion coefficient for bacterial cells (m\(^2\)/s). Diffusion coefficient is calculated with the following equation:

\[
D = \frac{k \times T}{6\pi \times \mu \times a_p} \tag{7}
\]

Where \( k \) is Boltzmann constant (J/K), \( \mu \) is kinematic viscosity (kg.m\(^{-1}\).s\(^{-1}\)) and \( T \) is temperature (K) at which the adhesion experiments are conducted.

The calculated \( Pe \) for all bacteria is 0.3, which is smaller than 1, indicating that diffusive mass transfer is larger than convective mass transfer for bacterial cells in the flow cell under experimental flow conditions.
Sherwood number is calculated for all of the adhesion experiments. Number of cells adhered to a surface was recorded and counted every minute for 30 min. The slope \((m)\) between the number of cells and time was calculated and was used in \(Sh\) calculation. \(Sh\) is a dimensionless parameter used to quantify the adhesion of bacterial cells to the surface. \(Sh\) can be calculated from the following equation (Song and Elimelech 1995):

\[
J = \frac{m}{A}
\]  

(8)

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

(9)

Where \(J\) is the flux of bacterial cells (#/m\(^3\).s), \(A\) is a microscope viewing area (m\(^2\)), \(a_p\) is the spherical equivalent diameter of bacterial cells (m), \(C_0\) is the bacterial initial concentration (#/m\(^3\)), and \(D\) is diffusion coefficient of bacterial cells (m\(^2\)/s).

To support the result from epifluorescence microscope, CLMS is also used to image adhered cells on 8-week, 11-week and treated groundwater biofilms. Total number of cells is counted from 3D-reconstructed CLMS images. \(Sh\) is calculated based on the following equation:

\[
J = \frac{N}{A \times t}
\]  

(10)

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

(11)
Where \( J \) is the flux of bacterial cells (\#/m^3.s), \( A \) is a microscope viewing area (m^2), \( t \) is time used in each adhesion experiment (30 min) (min), \( \rho \) is the spherical equivalent diameter of bacterial cells (m), \( C_0 \) is the bacterial initial concentration (\#/m^3), and \( D \) is diffusion coefficient of bacterial cells (m^2/s).

**Bacterial Cell Preparation and Characterization.** In our studies, 3 different strains of bacteria were used in the adhesion experiment. This includes laboratory strain *E. coli* S17, environmental strain *E. coli* 14f, and pathogenic strain of *Legionella pneumophila* (ATCC 33152). The environmental strain *E. coli* 14f was isolated by Ya Zhang from Newmark groundwater at University of Illinois at Urbana-Champaign. Both *E. coli*S17 and *E. coli* 14f cells were tagged with *gfp* plasmid. (Janjaroen et al., 2013) Luria Broth supplemented with 5 µg/ml carbenicillin was used to grow *E. coli* 14f and *E. coli* S17 at 37 °C for 8 hours at shaking speed of 200 rpm, and *gfp* was further induced with IPTG for 2 hours with the same incubating condition. A detail of the procedure can be found in our previous work. (Janjaroen et al., 2013) *Legionella* cells were electroporated with *gfp* plasmids pBG307 (Chen et al. 2006). *Legionella* cells were cultured in buffered yeast extract (BYE) rich media with supplement of 10 µg/mL chloramphenicol for 48 hours at 37 °C. Cells were harvested and cleaned in deionized water 3 times at 17,000 ×g. All bacterial cells were prepared fresh for each adhesion experiment. For further adhesion experiment, cells were re-suspended in KCl solution (3-300 mM) which was buffered with 1mM NaHCO_3 at pH 8.2-8.5. The final cell concentration for adhesion experiment was (1-5) ×10^7 cells/mL.

Viability of all the bacterial cells were checked with LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012, Invitrogen, CA, USA). The kit is comprised of SYTO 9®
dye, which generally stains all cells in a population that have intact membrane, while propidium iodide (PI) penetrates into cells with compromised membranes. To prepare a dye mixture, SYTO 9 and PI were diluted 200 times in DI water and mixed. The mixture was added to a cell solution containing \((1-3) \times 10^3\) cells/mL in DI water and was kept in dark at room temperature for 20 min. Unbound dye was washed with DI water subsequently. Stained cells were filtered through a black polycarbonate membrane filter (GTBP02500, EMD Millipore, MA, USA). Green and red cells adhered on the membrane were taken with 100× objective of epifluorescence microscope (Axio Observer.Z1, Zeiss, Germany) equipped with FITC (494/521 nm for green) and Cy3 (550/570 nm for red) filters. Twenty images of 100 cells were analyzed for green and red. The live and dead ratio was calculated based on red signal over the total cells (green + red).

Electrophoretic mobility of cells was measured using Zetasizer Nano (ZS90, Malvern, PA, USA). Experimental detail can be found in our previous work (Janjaroen et al., 2013). Briefly, cells were cleaned 3 times by centrifugation with DI water and resuspended in KCl solution (3-300 mM) at a concentration of \(10^7\) cells/mL. Cell surface hydrophobicity was measured by 2 methods; contact angle, and Microbial Adhesion to Carbon (MATH) test described in Walker et al., 2005. Contact angle of bacterial cells were measured by goniometer using diiodomethane as a probing solution (Janjaroen et al., 2013). For the MATH test, cleaned cells were added to 4 ml phosphate-buffered saline (PBS) at a concentration of \(10^8\) cells/ml, and 4 µL n-hexadecane was added this suspension. The mixture was vortexed for 2 min. Then the solution was left to sit still at room temperature for 20 min before samples were taken from each layer. Number of partitioning cells in water and hydrocarbon phase was counted with phase contrast
microscope (Axio Observer.Z1, Zeiss, Germany). Hydrophobicity was reported as a percentage of cells in hydrocarbon over total cells.

**Adhesion Experiment and Sherwood Number Calculation.** Adhesion of bacterial cells on different types of biofilms was conducted in a PFPC (FC 70, BioSurface Technologies Corp, MT, USA) under a flow rate of 1 mL/min to maintain a laminar flow within the chamber at ionic strength of 3-300 mM. Cell concentration was maintained at ~10^6-10^7 cells/mL. The focus was kept on the top surface of biofilms surface. Adhered bacterial cells were imaged and counted with an inverted epi-fluorescence microscope (Leica DM15000 M; Germany) every 1 min for 30 min. Detailed experiments can be found in our previous work. (Janjaroen et al., 2013) Adhesion kinetics were expressed by Sherwood number (Sh), which were subsequently used to compare results from different experimental conditions. Sherwood number calculation can be found in the previous section.

**Adhesion Experiment by Confocal Laser Scanning Microscopy (CLSM).** To ensure that adhered cells on biofilms did not entrap in the biofilm matrix, CLSM (TCS SP2 RBB, Leica, Germany) was used to image biofilms with deposited cells. After 30 min of adhesion experiment, cell-free electrolyte solution was used to wash unadhered cells. Then, biofilms were placed face down on a glass cover slip, and 20× objective was used to visualize the cells. GFP filter (488/513 nm) and 488 nm laser line were selected for this experiment. Number of cells within biofilm matrix was imaged and counted for further Sh calculation. At least 7 locations were chosen for imaging.

**Statistical Analysis.** Statistical analysis was conducted for all biofilms and bacteria electrophoretic mobility, contact angle and Sherwood numbers in both
fluorescence microscope and CLSM adhesion experiments. One-way ANOVA was used to analyze the similarity between electrophoretic mobility for each biofilm type based on 3-6 replicates \((p<0.05)\). For contact angle analysis, more than 8 replicates were analyzed. For comparing adhesion experiment between CLSM and fluorescence microscope, t-test (two tails test with unequal variance) was conducted for at least 3 replicates. As well, Linear Regression was used to test the independence between \(Sh\) number and IS. All one way ANOVA tests were conducted in Origin Pro 8.6, while t-test was tested in Microsoft Excel 2012. Results were reported as a \(p\) value of 95% confidence.

3.5. Results and Discussion

**Escherichia coli and Legionella Properties.** Electrophoretic mobilities (EPMs) of the studied bacterial strains were measured in solutions containing from 3 mM to 300 mM KCl at pH 8.3 as a means to determine the cell surface charge. At these experimental conditions, the EPMs of a laboratory strain *E. coli* S17, an environmental *E. coli* 14f, and *Legionella* were negative (Figure 3.3). For all three strains, EPMs became less negative with increasing ionic strength. Specifically, the EPMs of *E. coli* S17 were \(-3.3\pm0.1 \mu m. \text{s}^{-1}/(\text{V}.\text{cm}^{-1})\) and \(-0.8\pm0.1 \mu m. \text{s}^{-1}/(\text{V}.\text{cm}^{-1})\), while the EPMs for *E. coli* 14f were \(-2.5\pm0.04 \mu m. \text{s}^{-1}/(\text{V}.\text{cm}^{-1})\) and \(-0.8\pm0.09 \mu m. \text{s}^{-1}/(\text{V}.\text{cm}^{-1})\) at 3 and 300 mM, respectively. For *Legionella*, the EPMs were \(-1.98\pm0.09 \mu m. \text{s}^{-1}/(\text{V}.\text{cm}^{-1})\) and \(-0.45\pm0.08 \mu m. \text{s}^{-1}/(\text{V}.\text{cm}^{-1})\) at 3 and 300 mM, respectively. The decrease in the EPMs of all strains in KCl solution was due to the compression of electrical double layer thickness around the cells (Elimelech et al., 1998). Moreover, the EPMs of *E. coli* S17 were more negative than *E. coli* 14f and *Legionella* cells at IS ranging from 3 to 70 mM.
For example, the EPMs of *E. coli* S17, *E. coli* 14f, and *Legionella* were -3.3±0.1 μm. s⁻¹/(V.cm⁻¹), -2.5±0.04 μm. s⁻¹/(V.cm⁻¹), and -1.98±0.09 μm. s⁻¹/(V.cm⁻¹) at 3 mM, respectively. However, at higher IS than 70 mM, all strains had almost the same negatively charged.
Figure 3.3. Electrophoretic mobility of biofilms and 3 different bacterial strains as a function of KCl concentration ranging from 3 to 300 mM. All experiments were conducted at pH 8.2-8.5, at 25°C.
Both *E. coli* S17 and 14f cells were hydrophilic with diiodomethane contact angles of 70.6° ±2.2° and 88.3° ±3.5°. Our result was in a contact angle range (41°-97°) compared to other bacterial species using diiodomethane as a probe (Sharma and Hanumantha Rao, 2003; van der Mei et al., 1998). Due to safety precaution, *Legionella* cells were not allowed in a laboratory where the goniometer for contact angle measurement was situated. Therefore, hydrophobicity of *Legionella* cells was evaluated by the MATH test instead. The percentage of cells partitioning in hydrocarbon phase was 0%, 8% and 14% for *E. coli* 14f, *E. coli* S17, and *Legionella* cells, respectively. Thus, *Legionella* cells exhibited the most hydrophobicity among all of the bacterial strains.

The width and the length of both fluorescent *E. coli* and *Legionella* cells were measured by an epifluorescence microscope. The width and the length of *E. coli* 14f were 0.5±0.006 and 2.8±0.1 µm, which was corresponding to the equivalent radius of rod shape to sphere of 1.1 µm. For *E. coli* S17 and *Legionella* cells, the equivalent radii of rod shape to sphere were 1.06 µm and 0.87 µm, respectively. Live and dead ratio suggested that more than 90% of *E. coli* and *Legionella* cells used in adhesion experiments were intact.

**Properties of Biofilms With and Without Exposure to Disinfectant.** EPMs of groundwater biofilms and NH₂Cl-treated biofilms were shown in Figure 3.3-B, and EPMs of dechlorinated tap water and tap water biofilms were plotted in Figure 3.3-C. All biofilm samples had negative EPMs in solutions containing 3-300 mM KCl at pH 8.3. For example, the EPMs of 2-week and 8-week biofilms were -1.1±0.2 and -1.3±0.2 µmV⁻¹s⁻¹ cm⁻¹, respectively. Biofilms became slightly less negatively charged when biofilms aged.
Specifically, 20-week biofilms were \(-1.7\pm0.1 \, \mu \text{mV}^{-1} \text{s}^{-1} \, \text{cm}^{-1}\), while 2-week biofilms were \(-1.1\pm0.2 \, \mu \text{mV}^{-1} \text{s}^{-1} \, \text{cm}^{-1}\). Monochloramine treated biofilms were more negative than 20-week untreated biofilms. The same trend was observed for tap water treated biofilms. Specifically, 12-week biofilms treated with tap water had EPMs of \(-2.1\pm0.2 \, \mu \text{mV}^{-1} \text{s}^{-1} \, \text{cm}^{-1}\), while the EPMs of 20-week biofilms grown in dechlorinated tap water was \(-1.8\pm0.2 \, \mu \text{mV}^{-1} \text{s}^{-1} \, \text{cm}^{-1}\). Biofilm surface charge became more negative after the disinfection treatment may be due to oxidation of the biofilm function groups upon exposure to chlorine and monochloramine.

The hydrophobicity of different biofilm types was determined by diiodomethane contact angle measurement as shown in Table 3.1. Biofilms became more hydrophilic with increasing age. For example, contact angle of PVC, 2-, 8-week biofilms were 49.8º±2.2º, 56.5º±4.7º, 60.0º±4.8º, respectively. However, the hydrophobicity of biofilms increased at age of 20 weeks (46.3º±1.2º). However, groundwater biofilms treated with NH\(_2\)Cl did not show a significant change in surface hydrophobicity after the disinfection process (\(p>0.05\)). Similarly, hydrophobicity of biofilms treated with tap water for 12 weeks did not significantly change (\(p>0.05\)).
Table 3.1. Contact angle of diiodomethane on different types of biofilms. MATH test of laboratory strain *E. coli* S17, environmental isolated *E. coli* 14f, and *Legionella* shown as a percentage of cells suspended in hydrocarbon phase. Thickness and roughness of biofilms calculated from images taken by OCT method or CLSM. For CLSM, COMSTAT was used in analyzing pictures.

<table>
<thead>
<tr>
<th></th>
<th>( \Theta_{\text{diodomethane}} ) (% in hexadecane)</th>
<th>Average Thickness (( \mu \text{m} ))</th>
<th>Relative roughness coefficient (( R_a ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OCT</td>
<td>CLSM</td>
<td>OCT</td>
</tr>
<tr>
<td>PVC</td>
<td>49.8(^\circ) ± 2.2(^\circ)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-wk GW Biofilms</td>
<td>56.5(^\circ) ± 4.7(^\circ)</td>
<td>-</td>
<td>4.9±1.5</td>
</tr>
<tr>
<td>8-wk GW Biofilms</td>
<td>60.0(^\circ) ± 4.8(^\circ)</td>
<td>46±8.6</td>
<td>39.9±3.4</td>
</tr>
<tr>
<td>11-wk GW Biofilms</td>
<td>-</td>
<td>33±3.4</td>
<td>48.5±0.8</td>
</tr>
<tr>
<td>20-wk GW Biofilms (1(^{st}) batch)</td>
<td>46.3(^\circ) ± 1.2(^\circ)</td>
<td>98±12.19</td>
<td>-</td>
</tr>
<tr>
<td>20-wk GW Biofilms (2(^{nd}) batch)</td>
<td>-</td>
<td>99±10.04</td>
<td>-</td>
</tr>
<tr>
<td>12wk-NH(_2)Cl treated Biofilms (1(^{st}) batch)</td>
<td>43.8(^\circ) ± 4(^\circ)</td>
<td>99±8.79</td>
<td>-</td>
</tr>
<tr>
<td>12wk-NH(_2)Cl treated Biofilms (2(^{nd}) batch)</td>
<td>-</td>
<td>78±3.3</td>
<td>65±2.2</td>
</tr>
<tr>
<td>8wk-deCl(_2) tap water Biofilms</td>
<td>56.2(^\circ) ± 1.3(^\circ)</td>
<td>-</td>
<td>19.4±2.6</td>
</tr>
<tr>
<td>20wk-deCl(_2) tap water Biofilms</td>
<td>50.2(^\circ) ± 2.3(^\circ)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12wk-tap water treated Biofilms</td>
<td>53.7(^\circ) ± 1.9(^\circ)</td>
<td>-</td>
<td>6.1±0.5</td>
</tr>
</tbody>
</table>

**E. coli** S17

70.6\(^\circ\) ± 2.2\(^\circ\)

(7±2)\%

**E. coli** 14f

88.3\(^\circ\) ± 3.5\(^\circ\)

(0±0)\%

**Legionella**

(14±5)\%
Note: * represents relative roughness coefficient ($R_a$) of a clean PVC surface measured by profilometer.

Both CSLM and OCT methods were used to determine biofilm roughness, and the result is presented in Table 3.1. Biofilm roughness decreased with disinfection treatment ($p<0.05$). Specifically, 20-week biofilms had relative roughness coefficient of $0.47\pm0.09$ while the roughness of 12-week NH$_2$Cl treated biofilms decreased to $0.27\pm0.09$. Because the biomass of the biofilms treated with tap water was not enough to be detected with OCT, live/dead stain coupled with confocal microscope was used instead.

CLSM images and COMSTAT analysis revealed that the thickness of 2-week groundwater biofilms was $4.8\pm1.5$ µm, and the thickness increased to $39.9\pm3.4$ µm for 8-week biofilms (Table 3.1). Young biofilms were rougher than older biofilms, perhaps because of the influence of rough PVC surface on the young biofilm. Specifically, $R_a'$ of 2-week biofilms was $1.39\pm0.03$, while $R_a'$ for 8-week biofilms was $0.18\pm0.14$ (Table 3.1).

Biofilm structure analysis from OCT and CLSM was compared. Both techniques gave comparable biofilm average thickness. Specifically, average thickness of 11-week GW biofilms measured by OCT and CLSM was $46\pm4.8$ µm and $48.5\pm0.8$ µm, respectively (Table 3.1). However, the relative roughness coefficient analyzed by these two techniques was different. Relative roughness coefficient of 8-week biofilms measured by OCT was $0.55\pm0.04$ while by CLSM the roughness was $0.18\pm0.14$. Conversely, relative roughness coefficient of 12-week NH$_2$Cl treated groundwater
biofilms measured by OCT and CLSM increased from 0.15±0.04 to 0.26±0.08, respectively.

Monochloramine treated biofilms showed lower percentage of live to dead ratio than biofilms without exposure to monochloramine. Specifically, 11-week groundwater biofilms had live/dead ratio of 87%, while this ratio decreased to 46% for biofilms treated with NH$_2$Cl for 12 weeks. For biofilms grown with dechlorinated tap water for 8 weeks, live/dead ratio was 52%. The live/dead ratio of 12-week tap water treated biofilms could not be analyzed due to very thin biofilm allowing strong background signal interference.

However, though the roughness of treated biofilms decreased, the thickness of these biofilms remained the same except for tap water treated biofilms (Table 3.1). Chlorine used in biofouling control of polyamine RO membrane was found no to completely remove biofilms from the membrane at low shear force (Yu et al., 2013). However, other studies showed that biofilms treated with either monochloramine or chlorine have lesser thickness than non-treated ones (Ling and Liu, 2013; Xue and Seo, 2013). For example, biofilms of *P. aeruginosa* were cultivated over the course of 2 hours and were subject to chlorine for 6 days. However, *P. aeruginosa* biofilms grown from rich media have much higher roughness compared to 8 week groundwater biofilms studied here (relative roughness coefficients of 0.5 to 0.98 for *P. aeruginosa* biofilms vs. 0.18 for groundwater biofilm). Higher roughness observed for *P. aeruginosa* biofilms may allow for disinfectant to penetrate into the biofilm and contribute to slough off. Indeed, after exposure to disinfectant *P. aeruginosa* biofilms became rougher. Smoother groundwater biofilm studied here may be more compact preventing disinfectant penetration.
**Role of Ionic Strength on *E. coli* Adhesion.** Effect of ionic strength on adhesion kinetics of environmental *E. coli* 14f was investigated by observing the adhesion of cells on biofilms via fluorescence microscopy in KCl concentration ranging from 3-300 mM (Figure 3). Similar adhesion phenomenon on clean PVC and 2-week biofilm surfaces was observed. *Sh* of *E. coli* 14f on a clean PVC surface increased from 0.02±0.007 to 0.34±0.10 from 3 to 300 mM. In addition, the effect of IS on *E. coli* adhesion was observed on dechlorinated tap water biofilms treated with tap water for 12 weeks because of limited coverage of biomass on the PVC surface (Figure 3.4). The increased attachment of cells on PVC surface and PVC surface partially covered with 2-week groundwater biofilm or dechlorinated tap water biofilm was due to the compression of double layer thickness of the cells in high salt concentration, as observed before (Chen and Elimelech, 2006; Elimelech et al., 1998; Janjaroen et al., 2013; Liu et al., 2009; Redman et al., 2004). In contrast, the adhesion kinetics of *E. coli* 14f on mature biofilms (> 8 weeks) was independent from IS (*p*>0.05) (Figure 3.4-A and -B). The unrelated effect of IS on the adhesion of *E. coli* cells on mature groundwater biofilms was also observed in our previous work (Janjaroen et al., 2013) suggesting that another mechanism may attribute to this phenomenon. The roles of biofilm roughness and hydrophobicity on adhesion will be discussed below.
Figure 3.4. Sherwood number of environmental *E. coli* 14f on a) groundwater biofilms and GW biofilms treated with NH$_2$Cl, b) dechlorinated tap water biofilms and deCl$_2$ biofilms treated with tap water as a function of KCl concentration. Open symbols represent normally grown biofilms while close symbols are for biofilms exposed to disinfectants. All adhesion experiments were conducted at pH between 8.2-8.5, at 25°C.
Complementing fluorescence microscope, CLSM was used to investigate the adhesion of *E. coli* cells to groundwater biofilms as a function of IS due to CLSM’s ability to image deeper inside biofilms (Vroom et al., 1999; Wood et al., 2002). The attachment of *E. coli* cells observed by CLSM was statistically higher (*p*<0.05) than that determined by epifluorescence microscopy, e.g. *Sh*=1.28±0.54 and 0.4±0.17 for 20-week GW biofilms at 3 mM KCl (Figure 3.5). This observation is in contrast with previous report that the adhesion of *L. pneumophila* cells on smooth biofilms measured by fluorescence microscopy and by CLSM was not different (Shen et al., in preparation). In Shen et al., study, for 14-week GW biofilms with the relative roughness coefficients *R*$_a$” of 0.27±0.07, and *Sh* from epifluorescence microscope was statistically the same as *Sh* observed by CLSM (*p*>0.05). However, the 20-week GW biofilms studied here was rougher with *R*$_a$” of 0.4±0.02. These rougher biofilms may exhibit greater difference in heights throughout the biofilm surface, leading to underestimation of deposition on biofilms by epifluorescence microscope. CLMS should be able to provide a more accurate assessment of cell deposition on rough biofilms.
Figure 3.5. Sherwood number of 3 different strains of bacteria as a function of relative roughness coefficient ($R_a$). A) Sherwood number from adhesion experiment in PFPC (with open symbol), and B) Sherwood number obtained from CLMS images (with closed symbol). Blue, red and black symbols represent *Legionella*, *E. coli* S17 and *E. coli* 14f, respectively. Circle is for PVC surface, triangle is for 2-week GW biofilms, hexagonal is for 6-week GW biofilms, diamond is for 8-week GW biofilms, square is for 11-week GW biofilms, upside down triangle is for 14-week biofilms, clover is for 20-week GW biofilms (1st batch), star is for 20-week GW biofilms (2nd batch), triangle is for 12-week GW biofilms treated with NH$_2$Cl (1st batch), left-sided triangle is for 12-week GW biofilms treated with NH$_2$Cl (2nd batch). All experimental conditions were measured at 3mM KCl, pH 8.2-8.5 at 25 C$^\circ$.

**Effect of Cell Hydrophobicity on Bacterial Adhesion.** Hydrophobicity of three bacteria strains obtained from MATH test suggested that *Legionella* cells were more hydrophobic than *E. coli* S17 and *E. coli* 14f (Table 3.1). Hydrophobicity of three
bacteria strains obtained from MATH test suggested that *Legionella* cells were more hydrophobic than *E. coli* S17 and *E. coli* 14f (Table 3.1). Higher adhesion of hydrophobic *Legionella* cells compared to both *E. coli* strains on hydrophobic PVC surface was observed at 3mM KCl. Specifically, $Sh$ of *Legionella*, *E. coli* S17 and *E. coli* 14f on PVC at 3 mM KCl was 0.02±0.005, 0.2±0.003, 0.0038±0.0017, respectively. *Legionella* cells exhibited the highest hydrophobicity at (14±5)%. This trend in deposition on PVC surface is in agreement with previous studies for *Bacillus* spores (Husmark and Rönner, 1992), *E. coli*, *Staphylococcus epidermidis* (Gilbert et al., 1991) on glass surfaces, and *E. coli* and *Enterococcus faecallis* on immobilized TiO$_2$ (Faria et al., 2013). However, on mature GW biofilms no clear trend was observed between hydrophobicity of bacterial cells and the adhesion. Specifically, mature biofilms became more hydrophobic than PVC surface, but hydrophilic *E. coli* 14f was found to adhere the most to these biofilms (11-week and 20-week GW biofilms in Figure 3a). Therefore, hydrophobicity is not a significant mechanism controlling adhesion of cells on mature biofilms, and the role of biofilm structure on *Legionella* and *E. coli* cell adhesion will be discussed.

**Role of Biofilm Structure on Bacterial Adhesion.** Sherwood number ($Sh$) of three bacterial strains on GW biofilms as a function of biofilm relative roughness coefficient ($R_a$) is shown in Figure 4a and 4b. For all three bacterial strains, adhesion observed by fluorescence microscope and CLSM increased as a function of biofilm roughness ($p<0.05$). Specifically, $Sh$ of *E. coli* 14f on 11-week and 8-week GW biofilms increased from 0.3±0.05 to 0.6±0.14, while the roughness changed from 0.25±0.04 to 0.55±0.04 (Figure 3.5). The correlation between cell adhesion and biofilm roughness
was also observed for the other two bacterial strains in this study. The higher adhesion of cells on rougher biofilms may be due to the fact that rough biofilms pertain to higher peaks or deeper valleys. This rough biofilm surface structure may create more surface area for bacterial cells to adhere (Shen et al., in preparation) allowing cells to deposit at a different depth (Darbha et al., 2012). Other studies also reported that higher retention of Cryptosporidium oocysts was observed on rough P. aeruginosa biofilms (DiCesare et al., 2012; Searcy et al., 2006). Our result again suggested the importance of biofilm surface roughness on bacterial adhesion.

Biofilms exposed to disinfectants (chlorine and monochloramine) for 12 weeks expressed a decrease in roughness (Table 3.1). Sh of E. coli 14f and E. coli S17 on 12-week disinfected biofilms decreased to 0.18±0.06 and 0.0029±0.002, respectively (Figure 3a). Although hydrophobicity of treated and non-treated biofilms were statistically the same (p>0.05) (Table 3.1), surface charge and roughness of biofilm treated with disinfectants decreased significantly (p<0.05) (Figure 3.5). Thus, the decrease in adhesion of all bacteria strains correlated with biofilm surface charge and biofilm relative roughness coefficient (R₉). Thus, disinfectant altered biofilm surface charge and roughness which subsequently led to a decrease in bacterial adhesion. Chlorination has been shown to defloculate bacterial floc by adversely affecting the adhesion ability of floc bacteria and subsequently decreasing the adhesiveness of the floc (Mascarenhas et al., 2004).

3.6. Conclusions

- The physical structure of biofilms governs the attachment of pathogenic bacteria.
For three strains of bacteria, including a pathogenic *Legionella*, an environmental *E. coli* 14f, and laboratory *E. coli* S17, their adhesion on biofilms was positively correlated with biofilm roughness.

- Biofilms treated with disinfectants such as chlorine and monochloramine exhibited change in surface roughness, but not thickness. The roughness of biofilms treated with disinfectants decreased, leading to reduced bacterial attachment. These results suggested that maintaining a required disinfectant residual concentration in premise plumbing pipe will not completely remove biofilm, but may help to reduce adhesion of pathogenic cells intruding into the system. The risk of biofilms harboring bacterial pathogens may be reduced with the usage of disinfectant.

- Cell surface hydrophobicity influenced the adhesion of cell on a clean, hydrophobic PVC surface. *Legionella* cells, which had the highest surface hydrophobicity adhered the most on PVC while hydrophilic *E. coli* 14f was found to adhere the least. Besides cell surface hydrophobicity, cell adhesion on a clean PVC and 2-week biofilm surface was also controlled by ionic strength of the solution due to the compression of double layer thickness.

### 3.7. Acknowledgement

This work is supported by EPA agreement number 83487001. DJ was supported by Royal Thai Government. Undergraduate assistant, Lance Langer is acknowledged for maintaining biofilm reactors.

### 3.8. References


CHAPTER 4

THE EFFECT OF CELL STARVATION ON ADHESION KINETICS OF *LEGIONELLA PNEUMOPHILA* ONTO PVC, COPPER, AND BIOFILMS

4.1. **Abstract**

Adhesion kinetics of fresh and starved *Legionella pneumophila* cells were studied on clean PVC and copper surfaces, and biofilms grown on PVC surfaces using a parallel flow plate chamber with a epifluorescence microscope. Biofilms were grown on a PVC surface with groundwater for 11 and 20 weeks and were later used in adhesion experiments. Biofilm structure was determined with optical coherent tomography (OCT), while PVC and Cu surface roughness was measured with a profilometer. Fresh *Legionella* cells were starved in low nutrient groundwater for 5 weeks. Adhesion kinetics of both fresh and starved *Legionella* cells on clean PVC and Cu surface increased with ionic strength, suggesting that adhesion is controlled by electrostatic interactions. However, higher adhesion of starved cells was observed on a clean PVC surface due to higher cell surface hydrophobicity after starvation but the opposite trend was observed on clean Cu surfaces. On 20-week biofilms, no change in cell adhesion was observed with and without starvation. These results suggested that cell surface hydrophobicity played an important role in *Legionella* adhesion on a clean surface such as PVC and copper. However, on established biofilms, roughness

4.2. **Introduction**
In 1974, Legionnaire’s disease was first identified. The disease caused a severe outbreak of pneumonia among the participants of the American Legion Convention in Philadelphia (Fraser et al., 1977). Since 1974, Legionnaire’s disease has been identified and reported throughout the world (McDade et al., 1979; Terranova et al., 1978). In 1994, six people were hospitalized and diagnosed with pneumonia after travelling on the cruise ship Horizon (C Genese, MJ Hung, 1994). Moreover, from 1994 to 2004, more than 4000 cases of legionellosis was reported in European countries (Bartram et al., 2007). *Legionella pneumophila* was found to be a main cause of the outbreak (Muder and Yu, 2002). This bacterium can cause 'legionellosis', bacterial infections that can range from mild illness to potentially fatal pneumonia.

*Legionella* is found ubiquitous in soil and natural aquatic environment (Bartram et al., 2007; Fliermans et al., 1981). The bacteria are acid tolerant and are found in low pH environment ranging from pH 2.7 to 8.3 (Anand et al., 1983; Sheehan et al., 2005). In addition, *Legionella* can withstand a wide range of temperatures up to 66 °C (Dennis et al., 1984). *Legionella* can enter drinking water pipe systems via many routes such as through pipe break, cross connection and back flow (Snoeyink et al., 2006). Once bacteria enter the pipe, they can attach to pipe surface and be harbored by biofilms especially in a premise plumbing section which has low disinfectant residual (Snoeyink et al., 2006). Furthermore, when *Legionella pneumophila* co-exist with biofilms, it was found to be resistant to heat and disinfectants (Storey et al., 2004).

Few studies have suggested that pipe materials have an influence on bacterial adhesion (Niquette et al., 2000; Simões et al., 2007). For example, densities of bacteria on PVC surface were found 10-40 times lower than on iron surface. Moreover,
starvation of bacterial cells may lead to change in surface hydrophobicity and subsequently affect bacterial attachment on a surface (Husmark and Rönner, 1992; van Loosdrecht et al., 1987). However, the effect of both drinking water pipe materials as well as cell starvation on bacterial adhesion in different ionic strength has not been investigated. Therefore, our study aims to investigate the adhesion kinetics of fresh and starved *Legionella pneumophila* on PVC, copper and biofilms surfaces in different monovalent salt concentration. Hydrophobicity and surface charge of *Legionella* cells as well as the collector will be determined. Surface roughness of PVC and copper will be measured by profilometer, while optical coherence tomography will be used to identify biofilm roughness. The effect of cell hydrophobicity and collector surface roughness are investigated and discussed.

### 4.3. Materials and Methods

**Bacterial Cell Preparation and Characterization.** *Legionella pneumophila* (ATCC 33152) was used in the adhesion experiment. *L. pneumophila* cells electroporated with *gfp* plasmids pBG307 (Chen et al. 2006) were cultured in buffered yeast extract (BYE) rich media with supplement of 10 µg/mL chloramphenicol at 37 ºC for 48 hours. Freshly grown cells were harvested and cleaned with sterile deionized water (DI) by centrifugation at 17,000 ×g for 3 times prior to future experiments. For adhesion experiment, clean cells were re-suspended in KCl solution (3-300 mM) buffered with 1 mM NaHCO$_3$ at pH 8.2-8.5. The final cell concentration for adhesion experiment was (1-5) ×10$^7$ cells/mL.

Fresh and clean *Legionella* cells were further kept in sterile Newmark groundwater (University of Illinois at Urbana-Champaign) for starvation experiment.
Groundwater was filtered sterile with 0.22 µm filter (SCGVU01RE, EMD Millipore, MA, USA), and was subsequently disinfected with UV light at 250V for 10 min. Clean *Legionella* cells were re-suspended in prepared groundwater at final concentration of (1-5) ×10^10 cells/mL in a sterile 50-mL centrifuge tube. Tube was kept at static condition in dark at room temperature for 5 weeks for starvation.

Viability of both fresh and starved *Legionella* cells was carried out with LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012, Invitrogen, CA, USA). Intact cells (live) were stained with green dye (SYTO 9®), while membrane compromised cells were dyed with red dye (propidium iodide). Dye mixture was composed of an equal volume of SYTO 9® and propidium iodide diluted 200 times in DI water. 120 µL of mixture was added to 5 mL of cell solution containing (1-3)×10^3 cells/mL in DI water, and was incubated in the dark at room temperature for 20 min. Unbound dye was washed with 10 mL DI water before stained cells were filtered with black polycarbonate membrane filter (GTBP02500, EMD Millipore, MA, USA). The filter with adhered cells was carefully mounted on a glass slide with mounting oil in LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012, Invitrogen, CA, USA). Epi-fluorescence microscope (Axio Observer.Z1, Zeiss, Germany) equipped with FITC (494/521 nm for green) and Cy3 (550/570 nm for red) filters was used to observe and count green and red cells under 100× objective. Twenty images of 100 cells were analyzed for green and red. The live and dead ratio was calculated based on red cells over the total cells (green + red).

Electrophoretic mobility of fresh and starved cells was measured using Zetasizer Nano (ZS90, Malvern, PA, USA). Experimental detail can be found in our previous
work. (Janjaroen et al., 2013) Briefly, cells were cleaned 3 times by centrifugation with DI water and re-suspended in KCl solution (3-300 mM) at a concentration of $10^7$ cells/mL.

Both fresh and starved cells were subject to hydrophobicity measurement with Microbial Adhesion to Hydrocarbon (MATH) test described in Walker et al., 2005. Clean cells were suspended in 4 mL phosphate-buffered saline (PBS) at a concentration of $10^8$ cells/mL. 4 μL of n-hexadecane was added to this suspension and was mixed rigorously with vortexer for 2 min. The mixed solution was left undisturbed at room temperature to allow cells to partition into water and hydrocarbon phases. After 20 min, 10 μL of water was carefully withdrawn and pipetted into a disposable hemocytometer (DHC-N01, INCYTO, Korea). Number of cells was counted with a phase contrast microscope at 20× objective (Axio Observer.Z1, Zeiss, Germany). Hydrophobicity was calculated as a percentage of cells in hydrocarbon over total cells.

**Biofilm Growth in CDC Reactor.** Biofilms were grown on 2 surfaces; PVC and copper (Cu) in a CDC reactor according to previously described protocol (Janjaroen et al., 2013). Briefly, all reactors were fed with groundwater at 1.3 mL/min (HRT of 1.4 hr) and stirring speed in the reactor was at 125 rpm. Biofilms were grown for 11 and 20 weeks before taken out, analyzed, and used in adhesion experiments.

**Copper surface roughness controlling.** One-foot copper rod (8966K131, McMaster Carr, Illinois) was cut into 0.5-cm thick copper coupon. Freshly cut surface was smooth. Different roughness for copper surface was created with 2 types of sand paper. Smooth copper surface was kept non-abraded, while medium rough and rough surfaces were abraded with 210-grit and 50-grit sand paper (Forney, Texas).
Copper and Biofilm Imaging and Analysis. Thick groundwater biofilms were imaged and analyzed with Optical Coherence Tomography (OCT), which is only suitable for analyzing biofilms that exceed 2 µm in thickness (minimum resolution). Biofilms were placed in a parallel flow plate chamber in groundwater. Images were taken with the Spectral-Domain OCT system utilizing a mode-locked titanium:sapphire laser source (Kapteyn-Murnane Laboratories, Inc, Boulder, CO) centered at 800 nm with a bandwidth of 120 nm, providing an axial imaging resolution of 1.8 µm in water. Transverse resolution was 16 µm with the focus set several centimeters beneath the glass surface close to the biofilm structures. Cross-sectional images were acquired at 1 mm × 2 mm by an axial scan rate of 25 kHz. Image analysis program (Derlon et al. 2012) was used to process and analyze biofilm images for roughness and thickness. Calculation details are described below.

Image analysis consisted of the following steps:

(1) Detecting the membrane-biofilm interface (grey-scale gradient analysis);

(2) Binarizing the image (automatic thresholding);

(3) Calculating the physical properties of the biofilm: mean biofilm thickness ($\bar{z}$ in µm), absolute ($R_a$ in µm) and relative roughness ($R_a'$) coefficients.

These parameters were calculated according to the following equations:

$$
\bar{z} = \frac{1}{n} \sum_{i=1}^{N} z_i
$$

(1)
\[ R_n = \frac{1}{n} \sum_{i=1}^{N} \left( z_i - \bar{z} \right) \]  

(2)

\[ R'_n = \frac{1}{n} \sum_{i=1}^{N} \left( \frac{z_i - \bar{z}}{\bar{z}} \right) \]  

(3)

Where \( N \) is the number of thickness measurements, \( z_i \) is the local biofilm thickness (µm), and \( \bar{z} \) is the mean biofilm thickness (µm).

Roughness and thickness of clean PVC and Cu surfaces were analyzed with surface profiometer (Dektak 3030; Veeco; Plainview, NY, USA). A diamond tipped stylus of 12.5 µm was a scanning probe at the scanning rate of 1 µm/s. The vertical resolution at this scan rate was around 1 nm. Measurements were made by the sample stage moving underneath the diamond stylus. Surface variation caused the stylus to be moved vertically. According to this movement, electrical signals are changed from analog to digital form using an integrating A-D converter. Sample surface height and roughness are calculated based on equations used in OCT analysis.

Contact angle of PVC, Cu and biofilms was measured with diiodomethane using goniometer following our previous protocol (Janjaroen et al., 2013). Prior to the measurement, PVC and Cu surfaces were cleaned with 2% Hellmanex solution (9-307-010-507, Hellma GmbH & Co. KG, Germany) and rinsed 3 times with DI water. 12 measurements of contact angle were conducted for each surface for statistical analysis.

**Fluid Dynamics in a Parallel Flow Plate Chamber (PFPC).** Fluid dynamics and flow profile in the flow chamber was solved numerically with COMSOL; the Navier-
Stokes equation for incompressible, laminar flow was selected for this study. The initial velocity was calculated from the flow rate and cross-sectional area of flow. The boundary layer for both biofilm and glass surface was defined as ‘no slip’. Fluid velocity profile over biofilms and PVC surfaces were acquired after computation. Reynolds number \((Re)\) of the flow cell was calculated based on the characteristic length and flow rate of the flow cell (Bakker et al., 2003). Calculated \(Re\) \((Re = 1.24)\) was further used to check the validity of laminar flow assumption. Furthermore, the effect of flow rate on the change in biofilm roughness was also tested with higher flow rates. Shen et al. (publication in preparation) found that with rigid groundwater biofilms, higher flow rates did not significantly change biofilm structure determined by OCT. Calculation detail of \(Re\) and \(Pe\) number is listed below.

Reynold number \((Re)\) for the flow cell and a single bacteria cell was calculated to prove laminar flow regime using the following equations (Bakker et al., 2003; Clark, 2009).

\[
Re_{\text{cell}} = \frac{a_p \times V \times \rho}{\mu}
\]  

\(4\)

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

\(5\)

Where \(a_p\) is the spherical equivalent diameter of bacteria cells \((m)\), \(V\) is the flow velocity in the flow cell \((0.003 \text{ m/s in this study})\), \(\mu\) is the viscosity of the electrolyte solution used in this experiment \((\text{kg.m}^{-1}.\text{s}^{-1})\), \(Q\) is the flow rate in \(\text{m}^3/\text{s}\), \(w\) is the width of the flow cell chamber \((m)\), and \(b\) is the half depth between a coupon surface and a glass
cover slip (m). In our study, \( Re_{\text{Legionella}}, Re_{\text{E. coli}} \) and \( Re_{\text{flowcell}} \) are 0.005, 0.007 and 1.24, respectively.

Peclet number (Adamczyk and Van De Ven, 1981) was also determined for bacterial cell mass transfer in the flow cell. \( Pe \) represents the ratio between convection mass transfer and diffusion mass transfer (Clark’s book).

\[
P_{e_{\text{cell}}} = \frac{3 \times V \times a_p^3}{2 \times D \times b^2}
\]  

(6)

Where \( D \) if the diffusion coefficient for bacterial cells (m²/s). Diffusion coefficient is calculated with the following equation:

\[
D = \frac{k \times T}{6 \pi \times \mu \times a_p}
\]  

(7)

Where \( k \) is Boltzman constant (J/K) and \( T \) is temperature (K) at which the adhesion experiments are conducted.

The calculated \( Pe \) for all bacteria is 0.3, which is smaller than 1, indicating that diffusive mass transfer is larger than convective mass transfer for bacterial cells in the flow cell under experimental flow conditions.

**Adhesion Experiment and Sherwood Number Calculation.** Adhesion of *Legionella* cells on PVC, copper and GW biofilm surfaces was conducted in a PFPC (FC 70, BioSurface Technologies Corp, MT, USA). Cell concentration of \( \sim 10^6-10^7 \) cells/mL in ionic strength (IS) of 3-300 mM KCl was pumped into a flow cell with a flow rate of 1 mL/min. Pictures of adhered cells were taken with an inverted epifluorescence
microscope (Leica DM15000 M; Germany) every 1 min for 30 min. Detailed experiments can be found in our previous work. (Janjaroen et al., 2013) Adhesion kinetics were expressed by Sherwood number \((Sh)\), which were subsequently used to compare results from different experimental conditions. Sherwood number calculation is described below.

Sherwood number is calculated for all of the adhesion experiments. Number of cells adhered to a surface was recorded and counted every minute for 30 min. The slope \((m)\) between the number of cells and time was calculated and was used in \(Sh\) calculation. \(Sh\) is a dimensionless parameter used to quantify the adhesion of bacterial cells to the surface. \(Sh\) can be calculated from the following equation (Song and Elimelech 1995):

\[
J = \frac{m}{A}
\]

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

Where \(J\) is the flux of bacterial cells \((#/m^3.s)\), \(A\) is a microscope viewing area \((m^2)\), \(a_p\) is the spherical equivalent diameter of bacterial cells \((m)\), \(C_0\) is the bacterial initial concentration \((#/m^3)\), and \(D\) is diffusion coefficient of bacterial cells \((m^2/s)\).

**Chemistry Analysis of Groundwater.** Newmark groundwater filtered through green sand filter (Bradley et al., 2011) was collected for chemistry analysis. Inductively coupled plasma (ICP-MS) at School of Chemical Sciences, UIUC was used to analyze calcium, iron, magnesium and manganese. Hardness and alkalinity were tested with
alkalinity test kit (AL-DT, Hach, CO, USA). Total organic carbon (TOC) was measured by analyzer (TOC-V CHP, SHIMADZU, Japan). Groundwater was pre-filtered with a 0.22 µm filter (SCGVU01RE, EMD Millipore, MA, USA). pH of groundwater was also monitored.

**Statistical Analysis.** Statistical analysis was conducted for all *Legionella* electrophoretic mobility, contact angle and Sherwood numbers in both fluorescence microscope and CLSM adhesion experiments. One-way ANOVA was used to analyze the similarity between electrophoretic mobility for each biofilm type based on 3-6 replicates (*p*<0.05). For contact angle analysis, more than 8 replicates were analyzed. Linear Regression was used to test the independence between *Sh* number and IS. All one-way ANOVA tests were conducted in Origin Pro 8.6, while t-test was tested in Microsoft Excel 2012. Results were reported as a *p* value of 95% confidence.

4.4. **Results and Discussion**

*Legionella* cell surface properties after starvation. The effect of fresh and starved *Legionella* cell surface properties on adhesion kinetics was studied on a clean PVC and Cu surface in salt concentration ranging from 3 to 300 mM. Fresh and starved cells electrophoretic mobility was statistically the same (*p*>0.05) at every ionic strength and became less negative with ionic strength due to the compression of double layer thickness (Elimelech et al., 1998) (Figure 4.1). However, cell surface hydrophobicity increased from (14±5)% to (29±2)% after 5-week starvation (Table 4.1). The increased in *Legionella* surface hydrophobicity after 5-week starvation is consistent with previous study on marine *Pseudomonas* sp. S9, *Aeromonas hydrophila* and *Escherichia coli* K12 (Ascencio et al., 1995; Kjelleberg et al., 1983; Saini et al., 2011). During the course of
starvation, in limited nutrient condition, higher production of extracellular polymeric substances (EPS) may lead to more hydrophobicity cell surfaces, as was observed for *Pseudomonas* sp. S9 (Kjelleberg et al., 1983). Despite the change in surface hydrophobicity, live and dead ratio of fresh and starved cells did not alter significantly ($p>0.05$).

**Figure 4.1.** Electrophoretic mobility of fresh and 5-week-starved *Legionella* cells as a function of KCl concentration (3-300 mM). All measurements were buffered with 1mM NaHCO$_3$ at pH between 8.2-8.5 and were conducted at 25 $^\circ$C. At least 3 replicates were measured for each condition.
Table 4.1. Characteristics of pipe materials: PVC, copper, and biofilms surfaces. Thickness and roughness of PVC and copper, and biofilms was measured with profilometer and OCT, respectively. Hydrophobicity was measured with contact angle of diiodomethane and MATH test. Live and dead ratio of fresh and starved *Legionella* cells was measured by *BacLight*™ Bacterial Viability Kit.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Average Thickness (µm)</th>
<th>$R_a$</th>
<th>$Sh$ at 3mM</th>
<th>Hydrophobicity as $\Theta_{\text{diiodomethane}}$ or % in hexadecane</th>
<th>Intact/damaged Membrane Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC</td>
<td>17.2±4.4*</td>
<td>0.72±0.16*</td>
<td>0.0079±0.0004*</td>
<td>0.02±0.005‡                        (49.8 ± 2.2)°</td>
<td>-</td>
</tr>
<tr>
<td>Smooth Copper</td>
<td>7.8±0.24*</td>
<td>0.50±0.19*</td>
<td>0.01±0.004*</td>
<td>0.0015±0.001†                       (71 ± 0.6)°</td>
<td>-</td>
</tr>
<tr>
<td>Medium Rough Copper</td>
<td>5.4±1.6*</td>
<td>0.65±0.30*</td>
<td>0.02±0.0005*</td>
<td>0.0078±0.0006‡                     (70.9 ± 0.9)°</td>
<td>-</td>
</tr>
<tr>
<td>Rough Copper</td>
<td>8±3.1*</td>
<td>1.53±0.3*</td>
<td>0.041±0.0046*</td>
<td>0.013±0.001†                        (70.4 ± 2.4)°</td>
<td>-</td>
</tr>
<tr>
<td>Oxidized Copper</td>
<td>6.8±0.14†</td>
<td>0.53±0.30*</td>
<td>0.061±0.0041*</td>
<td>0.0096±0.0034‡                      (53.5 ± 2.5)°</td>
<td>-</td>
</tr>
<tr>
<td>11-week GW biofilms</td>
<td>46±4.80†</td>
<td>0.25±0.05†</td>
<td>0.02±0.007*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20-week GW biofilms</td>
<td>99±10.04†</td>
<td>0.32±0.06†</td>
<td>0.04±0.003*</td>
<td>0.03±0.009‡                        (46.3 ± 1.2)°</td>
<td>-</td>
</tr>
<tr>
<td>Fresh <em>Legionella</em> cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(14±5)⁰</td>
<td>(72±5)%</td>
</tr>
<tr>
<td>Starved <em>Legionella</em> cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(29±2)%</td>
<td>(70±3)%</td>
</tr>
</tbody>
</table>

**Note:** * and † represent relative roughness coefficient ($R_a$) of a clean PVC surface measured by profilometer and OCT, respectively. + and ‡ represent fresh and starved *Legionella* cells used in adhesion experiment.
Role of ionic strength on adhesion of fresh and starved cells on PVC and Cu surface. Adhesion of fresh and starved cells on clean PVC and Cu surfaces regardless of copper surface roughness was observed to increase with ionic strength (Figure 4.2 and 4.4). Specifically, $Sh$ of fresh Legionella cells on PVC surface increased from 0.0079±0.0009 to 0.013±0.003, while on Cu surface $Sh$ changed from 0.01±0.0038 to 0.15±0.092 at 3 and 300 mM for both surfaces. The increase in adhesion of cells as a function of ionic strength was due to the compression of double layer thickness of Legionella cells (Elimelech et al., 1998). This phenomenon was well studied in many previous works (Chen et al., 2010; Chen and Elimelech, 2006; Janjaroen et al., 2010; Liu et al., 2009; Redman et al., 2004; Walker et al., 2005). However, adhesion of Legionella cells on established biofilms was found independent of ionic strength (Figure 4.3). Therefore, another mechanism may control the adhesion of cells on biofilms.

![Figure 4.2](image-url)  

Figure 4.2. Sherwood number of fresh and starved Legionella cells on clean PVC and copper surfaces. Blue circle represents PVC surface, while orange star represents copper surface. Open and closed symbols represent fresh and starved cells accordingly. All adhesion experiments were conducted at pH between 8.2-8.5, at 25 C°.
Figure 4.3. Sherwood number of fresh *Legionella* cells on PVC surface (open circle), 11-week groundwater biofilms (open brown triangle), 20-week groundwater biofilms (open green triangle), starved *Legionella* cells on 20-week groundwater biofilms (closed pink star) as a function of KCl concentration. All adhesion experiments were conducted at pH between 8.2-8.5, at 25 °C.
Figure 4.4. Sherwood number of fresh *Legionella* cells (a) and starved *Legionella* cells (b) on copper surface (star), oxidized copper surface (diamond), medium rough copper surface (pentagon) and rough copper surface (circle) as a function of KCl concentration. All adhesion experiments were conducted at pH between 8.2-8.5, at 25°C.

**Role of Legionella cell hydrophobicity on cell adhesion on clean PVC and Copper.** Fresh and starved *Legionella* cells were used in adhesion experiment to evaluate the effect of cell surface hydrophobicity on the adhesion. On PVC surface, $Sh$
of starved *Legionella* cells was higher than fresh cells at every ionic strength while lower adhesion of starved cells was observed on a hydrophilic copper surface (Figure 4.2). Specifically, at 3 mM on PVC surface, *Sh* of fresh cells was higher than starved cells at 0.0079±0.0009 and 0.02±0.0052, respectively. The similar trend was also observed at higher IS of 300 mM. The fact that bacterial cells become more hydrophobic when exposed to stressful environment has been studied before. For instance, starved *D. Marina* cells became more hydrophobic and subsequently adhere more on polystyrene surface than fresh cells (Shea and Smith-Somerville, 1994). This change in cell surface hydrophobicity as well as the reduction in cell volume after starvation enhances the survival ability of bacteria by enabling bacteria cells to adhere more on a glass surface (Kjelleberg and Hermansson, 1984). Furthermore, our adhesion result of fresh and starved cells on PVC and Cu surface suggested a positive correlation between hydrophobicity of the bacterial cell and a collector surface, which is in agreement with previous studies that the affinity between bacterial cells and a collector surface hydrophobicity plays an important role in cell adhesion. For instance, hydrophobic *E. coli* and *Staphylococcus epidermidis* cells as well as hydrophobic *Bacillus* spores tend to colonize better on siliconized glass than hydrophilic cells (Dawson et al., 1981; Gilbert et al., 1991; Husmark and Rönner, 1992; van Loosdrecht et al., 1987).

**Role of surface roughness on cell adhesion.** The adhesion of fresh and starved cells on 20-week biofilms was statistically the same at 3 and 300 mM (*p*>0.5) (Figure 4.3). This similarity in fresh and starved cells adhesion on biofilms may be due to the biofilm surface roughness. Specifically, *Sh* of fresh cells on 11-week and 20-week biofilms at 3 mM was 0.02±0.007 and 0.04±0.003, while the roughness of 20-week
biofilms ($R_a'$ of 0.32±0.06) was higher than 11-week biofilms ($R_a'$ of 0.25±0.05) (Table 4.1). The effect of surface roughness on cell adhesion was also observed on clean PVC and Cu surfaces at 3, 100 and 300 mM. For example, $Sh$ of clean cells on PVC and Cu at 3mM was 0.0079±0.0009 and 0.01±0.004, respectively. Rough copper surface ($R_a'$ of 1.29±0.61) may lead to higher adhesion of fresh *Legionella* cells than smoother PVC surface ($R_a'$ of 0.72±0.16). Moreover, the adhesion of both fresh and starved *Legionella* cells also increased with copper surface roughness. Specifically, $Sh$ of starved *Legionella* cells on smooth and rough copper surfaces increased from 0.0015±0.001 to 0.013±0.001. Previous studies have shown that surface roughness can influence adhesion of particles (Darbha et al., 2010; DiCesare et al., 2012; L R Hilbert et al., 2003). For instance, polystyrene latex beads were found to deposit more on rougher rock surface (Darbha et al., 2010). However, the higher adhesion on rough Cu surface was not observed for starved cells. Besides when cells became more hydrophobic after starvation, starved cells tended to adhere more on hydrophobic PVC surface.

### 4.5. Conclusion

- Electrostatic interaction played an important role in *Legionella* cells adhesion on clean PVC and copper surface regardless of cell surface hydrophobicity. The adhesion of both fresh and starved *Legionella* cells on PVC and Cu surface increases with ionic strength due to the compression of cell double layer thickness.

- *Legionella* cells become more hydrophobic after 5 weeks of starvation in low nutrient environment but cell surface charge remains constant. The increase in cell surface hydrophobicity leads to higher adhesion of starved cells on hydrophobic PVC surface. However, opposite trend is observed for clean hydrophilic Cu surface.
as starved cells adhere lesser on Cu surface.

- Surface roughness of a collector surface plays an important role in *Legionella* cells adhesion. On a clean surface, fresh *Legionella* cells adhere more on rough copper surface than on smoother PVC surface. In addition, biofilm roughness also enhances *Legionella* adhesion on rougher biofilms. For instance, *Sh* of *Legionella* cells on rough 20-week biofilms is greater than on smooth 11-week biofilms. This result suggested that surface structure of different pipe materials may help facilitate the attachment of bacteria on the surface.

### 4.6. Acknowledgement

DJ was supported by Royal Thai Government. We acknowledged Stephan A. Burdin at Materials Research Laboratory at University of Illinois at Urbana-Champaign for help with surface profilometer.

### 4.7. References


CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

Biofilm roughness played an important role in the attachment mechanism of laboratory strain *E. coli* S17, environmental *E. coli* 14f, and *Legionella pneumophila*. Surprisingly, adhesion of bacterial cells on rough biofilms was independent of solution chemistry conditions such as ionic strength and hardness. On established biofilms, all three bacterial strains adhered to biofilms as a function of biofilm surface roughness. This suggested that the roughness of biofilms controlled the adhesion trend of bacterial cells despite solution chemistry, and the adhesion of cells would increase as biofilms became rougher regardless of the specific strain of incoming bacterial cells. Contrarily, on clean PVC, copper, or nascent biofilm surfaces, the adhesion of *E. coli* S17, *E. coli* 14f and *Legionella* cells increased with increasing solution ionic strength. On these clean surfaces, the DLVO electrostatic force between bacterial cells and the surface was the sole factor that governed adhesion kinetics. The increase in adhesion due to the reduction in electrostatic force was independent of bacterial cell surface hydrophobicity. For instance, starved *Legionella* cells, which became more hydrophobic from starvation, also had more adherences on PVC and copper surfaces in high ionic strength. This result suggests that on clean pipe surfaces, the adhesion of bacterial cells depends on solution chemistry. Even though all three strains of bacterial cells deposited more on clean PVC and copper surfaces, among the three, *Legionella* cells adhered the most on the hydrophobic PVC surface due to *Legionella*’s higher cell surface hydrophobicity. Also, starved *Legionella* cells with increased hydrophobicity
attached more on the clean PVC surface than on hydrophilic copper surface, suggesting an importance of cell surface hydrophobicity on bacterial attachment on clean surfaces.

Biofilms have been shown to react with disinfectants, reducing disinfectant effectiveness (Chen and Stewart, 1996). In our study, monochloramine did not completely eradicate biomass in thick biofilms (thickness more than 80 µm), but rather eroded their surfaces leading to smoother surface structures. Consequently, this smoothness discouraged the adhesion of all three bacterial strains onto treated biofilms. Conversely, chlorine seemed to completely disinfect dechlorinated tap water biofilms, which led to lower adhesion of bacterial cells.

Our studies focused on the initial adhesion of bacterial cells, which is an important step leading to bacterial growth and eventual detachment of those cells to environment. By understanding the adhesion mechanisms of the cells to various types of surface will help us in designing a pipe surface as well as regulating disinfectant residuals. However, in the environment, there are several processes that occur after bacterial cells attach to biofilm or pipe surfaces. Several key aspects have not been investigated here and remain a challenge for future research.

1. The detachment of bacterial cells and biomass from biofilm matrices is a natural process that happens throughout a biofilm’s lifetime. This detachment is actually an important mechanism that allows biofilms to grow and survive. However, the re-detachment of attached cells, especially within the presence of biofilms may lead to more severe cell dispersal in the environment. Therefore, a
comprehensive study is needed to better understand how bacterial cells detach as well as biofilms themselves (Step 4 in Figure 1).

2. *Legionella* cell viability and infectivity after the attachment to biofilms is also of great importance. If the cells lose their infectivity after adhering to biofilm surfaces, then they would no longer pose a threat to public health. However, that may not be the case as observed in this study where starved *Legionella* cells were still about 50% as viable after being exposed to low nutrient conditions for 5 weeks. The viability of *Legionella* cells may also increase when cells co-exist with amoeba since these protozoans will provide protection and habitat for *Legionella* cells (Step 3 in Figure 1) (Dupuy et al., 2011). And *Legionella* cells can eventually lyse their host cells and redistributed to the environment again (Step 4 in Figure 1). Consequently, a more in-depth study of bacterial viability and infectivity after adhesion and detachment as well as interaction with amoeba hosts is required.

3. In real premise plumbing systems, clean pipe surfaces are rare due to colonization by biofilms, scaling, and corrosion of the pipe itself. The current study has shown that solution chemistry is not an important factor in controlling bacterial attachment to established biofilms. However, the effects which pipe corrosion and scaling have on bacterial attachment were not investigated. With the presence of metal fragments from corrosion or hardness scaling, biofilms may be able to create more complex structures, which in turn may affect the attachment of incoming bacterial cells. Future research into biofilm and bacterial interactions in these more complex systems is required.
Figure 5.1. The schematic diagram showing 4 main processes of interaction between bacterial cells and biofilms in premise plumbing system: 1) attachment of bacterial cells onto biofilms, 2) bacterial growth and multiplication within biofilms, 3) bacterial cells co-exist with amoeba hosts within biofilms and 4) detachment of bacterial cells into the environment.

References

APPENDIX

PERMISSION LETTER FROM WATER RESEARCH

ELSEVIER LICENSE TERMS AND CONDITIONS
Nov 25, 2013

This is a License Agreement between Dao Janjaroen ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier Elsevier Limited
The Boulevard,Langford Lane
Kidlington,Oxford,OX5 1GB,UK

Registered Company Number 1982084
Customer name Dao Janjaroen
Customer address 705 W. Green St. APT 9
URBANA, IL 61801

License number 3275980193701
License date Nov 25, 2013
Licensed content publisher Elsevier
Licensed content publication Water Research
Licensed content title Roles of ionic strength and biofilm roughness on adhesion kinetics of Escherichia coli on groundwater biofilm grown on PVC surfaces
Licensed content author Dao Janjaroen,Fangqiong Ling,Guillermo Monroy,Nicolas Derlon,Eberhard Mogenroth,Stephen A. Boppart,Wen-Tso Liu,Thanh H. Nguyen
Licensed content date 1 May 2013
Licensed content volume number 47
Licensed content issue number 7
Number of pages 12
Start Page 2531
End Page 2542
Type of Use reuse in a thesis/dissertation
Portion full article
Format both print and electronic
Are you the author of this Elsevier article? Yes
Will you be translating? No
Title of your thesis/dissertation Role of disinfectants and pipe materials on bacterial adhesion onto biofilms
Expected completion date Dec 2013
Rightslink Printable License
https://s100.copyright.com/App/PrintableLicenseFrame.jsp?publisherID...
INTRODUCTION
1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS
2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
   “Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER].” Also Lancet special credit - “Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier.”

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed Rightslink Printable License
   https://s100.copyright.com/App/PrintableLicenseFrame.jsp?publisherID...
infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

**LIMITED LICENSE**

The following terms and conditions apply only to specific license types:

15. Translation: This permission is granted for non-exclusive world English rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. Website: The following terms and conditions apply to electronic reserve and author websites:

**Electronic reserve**: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:

- This license was made in connection with a course,
- This permission is granted for 1 year only. You may obtain a license for future website posting,
- All content posted to the web site must maintain the copyright information line on the bottom of each image,
- A hyper-text must be included to the Homepage of the journal from which you are licensing
at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com, and

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:
All content posted to the web site must maintain the copyright information line on the bottom of each image, and the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier’s online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article’s Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only. A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com. All content posted to the web site must maintain the copyright information line on the bottom of each image. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal Rightslink Printable License
https://s100.copyright.com/App/PrintableLicenseFrame.jsp?publisherID...

4 of 5 11/25/2013 10:13 AM

/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**: v1.6

If you would like to pay for this license now, please remit this license along with your payment made payable to “COPYRIGHT CLEARANCE CENTER” otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK501167306.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center