The influence of biofilm structure and total interaction energy on pathogen retention by biofilm

Varunraj Sendamangalam

The University of Toledo
A Thesis

entitled

The Influence of Biofilm Structure and Total Interaction Energy on Pathogen Retention by Biofilm

by

Varunraj Sendamangalam

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Chemical Engineering

_________________________________________
Dr. Youngwoo Seo, Committee Chair

_________________________________________
Dr. Dong-Shik Kim, Committee Member

_________________________________________
Dr. Isabel Escobar, Committee Member

_________________________________________
Dr. Patricia Komuniecki, Dean
College of Graduate Studies

The University of Toledo

August 2012
An Abstract of

The Influence of Biofilm Structure and Total Interaction Energy on Pathogen Retention by Biofilm

by

Varunraj Sendamangalam

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Chemical and Environmental Engineering

The University of Toledo

August 2012

In an aquatic environment, the biofilm found on solid surfaces is constituted of attached microorganisms and excreted extracellular polymeric substances (EPS). The presence of biofilm in natural and engineered systems may facilitate the retention of pathogenic bacteria, which may pose a serious concern to public health. Understanding the factors that influence the retention of pathogenic bacteria in environmental biofilms is essential to identify infection sources and develop control strategies. Thus, this study investigated the retention of a surrogate pathogenic bacterium, Escherichia coliT, in Pseudomonas aeruginosa biofilms with varying EPS excreting capacities. The quantities of detained E. coliT in biofilms and the biofilm structural characteristics were assessed using confocal laser scanning microscopy paired with image analysis. Meanwhile, the total interaction energy between E. coliT and the individual P. aeruginosa biofilms was computed using the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. The results indicated the heterogeneity of biofilm, such as surface roughness, aided the temporary retention of E. coliT in the biofilms.
Additionally, the presence of divalent ions in the solution not only greatly enhanced the pathogen retention, but also increased the heterogeneity of biofilm structure to a great extent. Interpretation of the obtained results using the DLVO theory further indicated the surface characteristics of the biofilm induced by EPS production influenced the magnitude of the energy barrier that must be overcome for initial pathogen attachment. The DLVO analysis also showed the increased divalent ion concentration had an even greater impact on energy barrier reduction to facilitate pathogen retention.

**Keywords.** Biofilm, DLVO, Surface roughness, Biofilm structure, CLSM.
This thesis is dedicated to my family for their endless love, support and encouragement since the beginning of my studies.
Acknowledgements

With immense pleasure I express my gratitude to my advisor Dr. Youngwoo Seo for all his technical, moral and financial support throughout my research. Without his support and advice this thesis would not have come to an end successfully.

I would further like to thank Dr. Dong-Shik Kim and Dr. Isabel Escobar for their enormous help and suggestions during my thesis defense as committee members. I am also thankful to the Department of Chemical Engineering, University of Toledo, for giving me an opportunity to pursue my Master’s program. Many individuals worked with me and assisted me in my project. I would like to especially thank Dr. Mau-Yi Wu for helping me in data analysis and in designing my research. Lastly, I thank my lab mates and friends, Christopher Hessler, Kimberly Coburn, and Zheng Xue for their valuable suggestions, guidance and support in conducting my experiments. The financial support from NSF is greatly appreciated.

Most importantly, I am very grateful to my family for their support and good wishes throughout my study. They all play an important role in making my dreams come true. Last but not least I am also very grateful to my friends here in Toledo for their tremendous support and encouragement throughout.
# Table of Contents

Abstract ........................................................................................................................................ iii

Acknowledgements .................................................................................................................... vi

Table of Contents ..................................................................................................................... vii

List of Tables ............................................................................................................................ ix

List of Figures ........................................................................................................................... x

List of Abbreviations ............................................................................................................... xi

List of Symbols ......................................................................................................................... xii

1 Importance of Study ............................................................................................................. 1

2 Literature Review ................................................................................................................ 3

   2.1 Biofilm – Structure (Surface roughness, Surface-area-to-volume ratio) and Composition (EPS content) influence on the entrapment of pathogens .................. 4

   2.2 Electrical double layer compression, surface charge neutralization, and bridging effects on the interaction of pathogens with a substratum surface ................... 7

   2.3 Influence of Hydrophobic interactions in the adhesion of bacteria to a substratum surface ........................................................................................................ 10

   2.4 Use of DLVO theory for the calculation of adhesion energies between the substratum surface and pathogenic bacteria ......................................................... 11
3 Background and Scope of the work ......................................................................................... 14
4 Materials and Methods............................................................................................................. 18
  4.1 Bacterial strains and media ............................................................................................. 18
  4.2 Biofilm cultivation in a flow cell system ....................................................................... 19
  4.3 Pathogen interactions with preformed biofilms ............................................................ 20
  4.4 Biofilm image acquisition and analysis ........................................................................... 20
  4.5 Quantification of E. coli\textsuperscript{T} in the effluent ....................................................... 21
  4.6 Total interaction energy analysis using the DLVO theory ............................................ 22
  4.7 Statistical analysis .............................................................................................................. 26
5 Results and Discussion ......................................................................................................... 27
  5.1 E. coli\textsuperscript{T} retention in biofilms ....................................................................... 27
  5.2 Enumeration of E. coli\textsuperscript{T} in the effluent samples ........................................... 29
  5.3 EPS influence on biofilm structural characteristics ....................................................... 30
  5.4 E. coli\textsuperscript{T} retention in biofilms in the presence of divalent ions ..................... 32
  5.5 The influence of divalent ions on biofilm structure ....................................................... 33
  5.6 Correlation between biofilm structural parameters and E. coli\textsuperscript{T} retention ....... 36
  5.7 Total interaction energy analysis between E. coli\textsuperscript{T} and P. aeruginosa biofilm... 37
6 Conclusions ............................................................................................................................. 43
7 Recommendations for future research ................................................................................ 44
References ................................................................................................................................... 46
List of Tables

5.1 Structural characteristics of the biofilms cultivated in LB broth................................. 28
5.2 Structural characteristics of the biofilms cultivated in LB broth with divalent ions. . 33
5.3 Contact angle and surface free energy of tested bacteria and medium....................... 38
5.4 Free energy of van der Waals component and Hamaker constant of tested biofilm systems ............................................................................................................................. 38
5.5 Surface potentials of the bacteria................................................................................. 39
5.6 Profile of the DLVO parameters for interaction between the *P. aeruginosa* biofilms and *E. coli* in two different divalent concentrations ................................................................. 41
List of Figures

1-1 A graphical abstract of the study. ................................................................. 2

2-1 CLSM images of \textit{P. aeruginosa} biofilms grown without and with divalent ions .... 9

2-2 Stages of Biofilm formation ........................................................................... 12

3-1 \textit{Pseudomonas} strains with varying EPS secreting capacities. ...................... 16

3-2 Schematic depicting the investigation of different factors on the interaction of pathogens with biofilms undertaken in this study.............................................................. 17

4-1 Flow cell setup used for biofilm development. ......................................................... 19

5-1 Orthogonal views of a CLSM image of a six day old algT(U) biofilm with \textit{E. coli}^T. 28

5-2 FACS analysis of \textit{E. coli}^T retention in biofilms. ...................................................... 30

5-3 Quantity of \textit{E. coli}^T detained versus surface roughness coefficient ................. 37

5-4 Total interaction energy profiles between the \textit{E. coli}^T and biofilms ...................... 40
List of Abbreviations

CFU ................................................................. Colony forming unit
CLSM ............................................................ Confocal Laser Scanning Microscope
DLVO ............................................................. Derjaguin-Landau-Verwey-Overbeek
EPS .............................................................. Exopolymeric substance
FACS ............................................................. Fluorescence activated cell sorter
pH ................................................................. potential for hydrogen ion concentration
rpm ............................................................... rotations per minute
UV-vis .......................................................... Ultraviolet-visible
List of Symbols

mL ........................................................................................................ milliliter
K ..................................................................................................... Kelvin
C ..................................................................................................... Celsius
mg .................................................................................................. milligram
g .................................................................................................... gram
mm .................................................................................................. millimeter
nm .................................................................................................. nanometer
µm ................................................................................................. micrometer
J ................................................................................................... Joule
mJ ................................................................................................. milli Joule
mM ............................................................................................... millimolar
mol ................................................................................................. mole
Chapter 1

Importance of Study

The persistence of pathogenic bacteria in the environment has been a great concern to protect public health. In order to control the transport of pathogenic bacteria in an engineered environment, e.g. water distribution system; it is essential to understand their longevity and fate in the environment, especially during the initial interaction. In an aquatic environment, the surface that directly interacts with pathogenic bacteria is commonly covered by microbial biofilms, an amalgamation of attached microorganisms and excreted extracellular polymeric substances (EPS). The presence of a biofilm may facilitate the retention of pathogenic bacteria and subsequent proliferation. Even though the interaction between pathogenic bacteria and surfaces e.g. quartz and glass, has been investigated, the direct observation of the interfaces between pathogenic bacteria and biofilms in aquatic environments is still lacking. Furthermore, the detailed observation of the influence of a biofilm structure on pathogen interaction remains inconclusive.

Thus, this study will investigate the retention of a surrogate pathogenic bacterium, Escherichia coli, in Pseudomonas aeruginosa biofilms with various EPS excreting capacities. The gram-negative bacteria P. aeruginosa commonly found in soil, water, plants and animals is well known in causing major infections in immunocompromised
patients. It has been frequently used as a model organism to initiate biofilms in laboratory experiments. The polysaccharide (Alginate) is the primary EPS component present in the *P. aeruginosa* biofilms.

We believe this study will enhance an insight into the understanding of how pathogenic bacteria interact with the different biofilms grown with and without divalent ions. A graphical abstract depicting this study is shown in Figure 1.1.

![Graphical abstract of the study](image)

**Figure 1-1:** A graphical abstract of the study (Courtesy: Dr. Mau-Yi Wu).
Chapter 2

Literature Review

Research in the past has reported that the presence of a biofilm community in environmental and water distribution systems can significantly influence the propagation of associated pathogens within the biofilm. Biofilms present in drinking water distribution systems and porous media systems have shown to harbor various pathogenic microorganisms like *Erwinia chrysanthemi*, *Escherichia coli*, etc and protecting them from antibiotic treatment, and toxic chemicals thereby serving as environmental reservoirs of disease [1-7]. These pathogens can detach or slough off from the biofilm in drinking water systems eventually leading to various human infections. *E. coli*, a common pathogen found in food and water has been reported in causing various cases of food poisoning [8, 9] gastroenteritis [10], urinary tract infections [11] in humans leading to occasional product recalls. *E. chrysanthemi* is well known in causing soft rot, wilt, and blight diseases in a wide range of plant species [12]. Taking into consideration the public health risks caused by the infectious pathogens that get released as a result of biofilm detachment, it is very important to understand the influence of biofilm structure on short-term and long-term entrapment and retention of pathogen under static as well as flowing conditions.
Several studies that focused on biofilm-pathogen interaction research [3, 4, 6, 7, 13, 14] have reported the possible mechanisms involved in aiding the biofilm in the capture and retention of pathogens. The adhesion between the pathogens and biofilms may be governed by a variety of interactions namely:

I. Biofilm - Structure (Surface roughness, Surface-area-to-volume ratio, etc.) and Composition (EPS content) influence on the entrapment of pathogens.

II. Electrical double layer compression, surface charge neutralization, and bridging effects on the interaction of pathogens with a substratum surface.

III. Influence of Hydrophobic interactions in the adhesion of bacteria to a substratum surface.

2.1 Biofilm – Structure (Surface roughness, Surface-area-to-volume ratio) and Composition (EPS content) influence on the entrapment of pathogens

Biofilm structural parameters including the biofilm surface roughness and surface-area-to-volume ratio have shown to aid the biofilm in the capture and retention of a parasite – Cryptosporidium parvum oocysts [6, 7, 13]. The studies used environmental biofilms and wild type and mutant type P. aeruginosa strains viz. PAO1 and PDO300. In Searcy et al’s study, PAO1 and PDO300 biofilms were grown in different media (LB and Jensen’s) and their influence of biofilm structure and background media on the capture and retention of oocysts were investigated. The results showed a significant influence of the background media on the capture of oocysts as LB broth had a greater influence than Jensen’s media. Results also suggested that the heterogeneous nature of the biofilms characterized by the presence of pores and voids facilitated the capture and retention of
oocysts. Overall based on the findings, a positive correlation was observed between the oocysts captured and the biofilm structural parameters viz. surface roughness and surface–area-to-volume ratio. Though this study quantified the structure of different biofilms and influence of different media on oocyst deposition, the influence of divalent ions on the biofilm structure and oocyst entrapment was however not investigated.

*P. aeruginosa* biofilms present in porous media have been reported to influence the initial adhesion, growth and detachment of indicator bacteria *E. coli* JM109 [4]. This study reported the amount and composition of EPS of the two *P. aeruginosa* biofilms: PDO300 and PAO1 profoundly impacted the deposition and retention of *E. coli* in porous media. Among the two biofilms, PDO300 biofilm captured fewer *E. coli* despite that PDO300 biofilm was thicker and had a higher surface roughness. Results obtained in this study clearly indicated biofilm surface hydrophobicity and polymeric interactions played a more crucial role than the biofilm architecture and reduced bed porosity in controlling the distribution of *E. coli* within the biofilms. The primary difference in the study compared to the work detailed in this document is that there was no quantitative data to support the involvement of the biofilm structural parameters on *E. coli* retention within the porous media biofilms [4].

In addition to the investigation of influence of PDO300 and PAO1 porous media biofilms on the deposition and retention of *E. coli*, the adhesion and retention of a bacterial phytopathogen *Erwinia chrysanthemi* within the same porous biofilms were also investigated by Yang Liu and his colleagues [3]. The results indicated that the biofilm physical structure and polymeric interactions between biofilms and phytopathogen controlled the bacterial deposition within the biofilms. Based on the results, there was a
higher deposition of phytopathogens within the PAO1 biofilms when compared to the PDO300 biofilms and the hydration effect in PDO300 biofilms that incorporated a large amount of water into their structure through hydrogen bonding was believed to account for the interference and lower deposition of phytopathogen within the biofilm. Surface roughness was however not believed to play a role in the deposition of phytopathogens within the biofilms as contradicting results were obtained.

Helmi et al. [14] investigated the interactions of *Cryptosporidium parvum*, *Giardia lamblia*, Vaccinal Poliovirus Type 1, and Bacteriophages φX174 and MS2 with drinking water and wastewater biofilms. The results proposed were that a higher concentration of poliovirus was retained in the wastewater biofilm than the drinking water biofilm due to the presence of more voids and cavities in the wastewater biofilm structure. However, the attachment of *Giardia lamblia* and *Cryptosporidium parvum* oocysts were similar in both drinking water and wastewater biofilms as there was no influence of the cavities and voids on their attachment to the biofilms. The proposed explanation for the lower numbers of attachment of *Giardia lamblia* and *Cryptosporidium parvum* oocysts was due to their larger sizes when compared to the poliovirus.

The pathogen-biofilm interaction studies in the past focused on the use of *P. aeruginosa* PDO300 and PAO1 biofilms that are well known in overproducing and producing EPS in normal levels. However, influence of a biofilm structure without EPS content such as an algT(U) biofilm on the capture and retention of pathogens has not been presented before.
2.2 Electrical double layer compression, surface charge neutralization, and bridging effects on the interaction of pathogens with a substratum surface

The electrical double layer exists as a result of the dominance of negative charge molecules on the bacterial cell surface and the negative charge existing on the substratum surface. Divalent ions have shown to enhance the adhesion of bacterial cells to surfaces by compression of the electrical double layer [15]. Calcium ions in particular have shown to play an important role by involving in non-specific interactions such as neutralization of the electrical double layer between cell and the substratum surface. The repulsive force generated as a result of the negatively charged bacterial cell surface and the substratum surface hinders the attachment of bacterial cells to the substratum surface. It can be clearly understood that the distance between the cell surface and substratum surface influence the attractive forces during the reversible attachment phase; the shorter the distance the greater the attractive force. It is well known that the ionic strength of the medium plays a significant role in influencing the extent of adhesion of bacterial organism to the surface. The thickness of the electrical double layer is inversely proportional to the square root of the ionic strength or electrolyte concentration of the medium [16, 17]. Hence, when divalent ions are supplemented to the nutrient media, an increase in the electrolyte concentration of the medium can result that eventually compresses the electrical double layer. The DLVO theory indicates that this results in a reduction or elimination of the repulsive energy barrier [18, 19].

Surface charge neutralization is the second mechanism that can influence the interaction between a biofilm surface and pathogenic microorganisms. Lowering the surface charge of the interacting molecules can result in a drop of repulsive energy curve
that then allows the van der Waals forces to reduce the energy barrier. Charge neutralization can be easily monitored using zeta potentials of the bacterial cells. Ca$^{2+}$ ions have been reported to alter the properties by binding to other molecules [15]. Kuznar et al. [18] pointed out that the specific binding of Ca$^{2+}$ to surface functional groups of molecules resulted in surface charge neutralization that was clearly evidenced by the reduction in the absolute values of zeta potential. By surface charge neutralization, the divalent ions reduce the repulsive energy barrier between the pathogen and the substratum surface. Charge neutralization of the surface proteins in the presence of divalent ions resulted in conformational changes and subsequent collapse of the proteins eliminating the steric repulsion and thereby contributing to the higher oocyst attachment to the surface [18].

Bridging effect is one of the most important mechanisms, and is often used in conjunction with charge neutralization. Research has reported that divalent ions act as bridging ions by forming cationic bridges between the negatively charged bacterial surface and the negatively charged substratum surface. Calcium has been reported to facilitate the bacterial association among Streptococci in the oral cavity [20] by forming polymeric cationic bridges between the polymeric molecules in the individual bacterial cells. This mechanism of “bridging effect” has been proposed as a major factor in plaque formation. Reports have also shown that Ca$^{2+}$ ions stimulate the development of thick and compact biofilms with increased mechanical stability [21, 22].
The CLSM images (Figure 2-1) clearly show that there was a significant influence of divalent ions on the *P. aeruginosa* mucoid (alginate overproducing strain) biofilm structure. Without divalent ions, the biofilm structure was uneven and lumpy whereas in the presence of divalent ions, a thicker and a more stable biofilm structure was observed due to the bridging effect between the alginate molecules in the biofilm. Sarkisova et al. [23] observed a similar increase in the thickness from a 10 to 20 fold with a mucoid *P. aeruginosa* FRD1 biofilm. Kerchove et al. [24] showed that the adhesion of non-motile bacteria was governed by cation bridging interactions between high-affinity sites at the bacterial surface and either clean or alginate-conditioned substrate surfaces. Simoni et al. [25] found that Mg$^{2+}$ ions enhanced the deposition of *Pseudomonas* sp. strain B13 in sand columns with respect to a solution of identical ionic strength containing Na$^+$ ions. Their findings suggested that there was a specific binding of the divalent ions to the cell surface thereby influencing the deposition of the bacterial cultures to the substratum surface. However, the relationship between the concentration of divalent ions in the nutrient medium and the adhesion process has been reported to be complex [24].

![Figure 2-1: Confocal Laser Scanning Microscopy (CLSM) images of *P. aeruginosa* biofilms grown a) without divalent ions, and b) with divalent ions (Courtesy: M. Strathmann, IWW Water Centre, Germany)](image-url)
2.3 Influence of Hydrophobic interactions in the adhesion of bacteria to a substratum surface

Hydrophobic interactions are usually the strongest of all long-range non-covalent interactions and are defined as the attraction between apolar or slightly polar molecules, particles or cells, when immersed in water. Hydrophobicity has been reported to be a major driving force for the attachment of bacteria to different substratum surfaces [26, 27]. Experimental tests showed that the adhesion of *Alcaligenes denitrificans* to polypropylene (PP) surface was the strongest in comparison to other polymeric materials including high density polyethylene (HDPE), poly(vinyl chloride) (PVC) and polymethylmethacrylate (PMMA). PP was more hydrophobic than the other polymeric materials and therefore influenced the adhesion of higher numbers of bacteria [26]. The adhesion of *Staphylococcus epidermidis* a common etiological agent well known in causing infections in indwelling medical devices, such as catheters and intracardiac prosthesis to a variety of polymeric supports was also investigated in the study. A linear correlation was observed between the number of attached cells and surface hydrophobicity. Cellulose diacetate (CDA) was the most hydrophilic material and also the least adherent among the different polymeric materials used in this study. The results showed that when the hydrophilicity of the CDA polymeric surface was enhanced by chemical treatments, there was a decrease in the number of attached *S. epidermis* cells to the polymer surface thereby showing the importance of hydrophobic interactions in bacterial adhesion to a substratum surface. Dai et al. [27] reported that hydrophobic interactions significantly influenced the adhesion of *G. lamblia* cysts to solid surfaces. In their work, different polymers including aminosiloxane, fluorosiloxane and a cationic
polymer (polyquaternary ammonium resin) with different charge densities and hydrophobicities were used to create polymeric surfaces. The fluorosiloxane was moderately negatively charged and the most hydrophobic surface that contributed to the 100% attachment of the cysts to its surface.

2.4 Use of DLVO theory for the calculation of adhesion energies between the substratum surface and pathogenic bacteria

The DLVO theory has been widely used as a theoretical model not only qualitatively but also quantitatively to calculate the adhesion energies involved in the bacterial attachment to different substratum surfaces [28]. It is well known that the first stage (Figure 2-2), which is a reversible phase in the adhesion of bacteria to a substratum surface, is governed by non-specific physical and/or chemical interactions. The different non-specific forces involved are the van der waals forces, steric interactions and electrostatic double layer interactions. These three forces are collectively known as the DLVO (Derjaguin, Verwey, Landau and Overbeek) forces and the DLVO theory can be used to describe the net interaction energies involved between the cell and substratum surface during the initial reversible attachment phase [29, 30]. However, when hydrophobic energies are involved in the quantification of the interaction energies between the bacterial cells and substratum surface, an extended DLVO (XDLVO) theory comes into consideration [31, 32].
Liu et al. [33] studied the effect of oocyst surface macromolecules on oocyst-quartz surface interaction and oocyst adhesion. The results showed that the composition and conformation of oocysts surface macromolecules majorly affected all the interaction (van der Waals, double layer and steric-hydration) forces. One of the conclusions in this study was that when the oocyst surface was more negatively charged, and the electrical double layer force dominated the van der Waals force resulting in more repulsion between the oocyst and quartz surface.

Bayoudh et al. 2009 [28] used the DLVO and the extended DLVO theories to qualitatively and quantitatively predict the interaction energies between *Pseudomonas stutzeri* (PS) and *Staphylococcus epidermidis* (SE) to hydrophilic glass and hydrophobic indium tin oxide (ITO) coated glass surfaces at long and short distances. The findings observed were that the DLVO predictions showed that the adhesion was favorable for all bacteria-substrate combinations. The XDLVO theory predicted unfavorable bacterial adhesions to glass surface whereas with the ITO surface a favorable adhesion of PS bacteria and unfavorable adhesion of the SE bacteria was observed. However, the DLVO
theory has seldom been used to describe the interaction energies between biofilm surfaces and pathogenic bacteria.
Chapter 3

Background and Scope of the work

A Biofilm is a gelatinous matrix formed on a solid surface comprising microorganisms aggregated by excreted extracellular polymeric substances (EPS) [1]. The EPS are high molecular weight biopolymers consisting of polysaccharides, proteins, lipids, and nucleic acids, which may account for up to 50-90% of the total organic matter in a biofilm [34, 35]. EPS have been reported to play a pivotal role in promoting initial bacterial attachment, formation of micro colonies and development of a mature biofilm structure in a variety of environmental and engineered aquatic systems [35-37]. Additionally, the EPS excreting capacities of bacteria can affect the microstructures of the biofilm, which can significantly influence the retention of pathogenic bacteria in the environment [13, 38, 39]. If pathogens proliferate in the biofilm due to the EPS influence, it could raise a serious concern to public health [3, 40-42]. However, most studies have reported pathogen interaction with an indigenous biofilm, without considering the variability in biofilm structural characteristics on pathogen retention [6, 13]. These features may be critical as the retention of pathogenic bacteria in a biofilm involves multiple interconnected physiochemical properties which include surface charge and hydrophobicity of the biofilm [43], as well as the physical interaction between the
pathogen and biofilm mediated by the surface structure of the pathogens (e.g. pili or flagella) [3, 4, 44].

Beyond the intrinsic characteristics of the biofilm, the aquatic chemistry can also significantly influence the attachment and retention of pathogen on the biofilm surface. Electrostatic forces influenced by the pH and ionic strength of solution will either facilitate or hinder pathogen retention [13, 45, 46]. Alternatively, divalent ions such as calcium or magnesium are shown to serve as bridging ions that can stabilize the negatively charged biofilm EPS and thus influence the biofilm formation as well as subsequent pathogen interaction [24, 47, 48].

While the retention of pathogenic bacteria by biofilm in aquatic environment involves biofilm characteristics and aquatic chemistry, few studies have examined these interactions from both perspectives [13, 33, 49]. Thus, this study investigated the influence of biofilm structure and divalent ions (Ca$^{2+}$, Mg$^{2+}$) on the initial retention of pathogenic bacteria in the biofilm. Three Pseudomonas aeruginosa strains (Figure 3-1) with varied EPS secreting capacities were employed as model environmental bacteria to develop confluent biofilms, where the influence of biofilm structure and composition was evaluated on initial pathogen retention using confocal laser scanning microscope (CLSM) coupled with image analysis. Escherichia coli$^{T}$ was used as a surrogate pathogen to investigate its interaction with the preformed microbial (P. aeruginosa) biofilms in a simulated aquatic environment.
Meanwhile, the influence of divalent ions on pathogen retention was also investigated by cultivating the biofilm in different divalent concentrations. Based on the obtained results, the initial bacterial retention behavior in biofilms were interpreted by comparing and correlating biofilm structural characteristics and retained pathogen quantities, as well as the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory was utilized to understand the interaction energies between bacteria and biofilms in aquatic environment [28, 33, 45, 50].
Figure 3-2: Schematic depicting the investigation of different factors on the interaction of pathogens with biofilms undertaken in this study.

Considering the lacking work in the biofilm-pathogen research done in the past, this project was designed to answer the lacking questions and also investigate multiple factors on the interactions between pathogenic microorganisms and preformed biofilms. The schematic (Figure 3-2) above represents the various factors that will be investigated on the interaction of pathogenic microorganisms with preformed biofilms.
Chapter 4

Materials and Methods

4.1 Bacterial strains and media

Three *Pseudomonas aeruginosa* strains (wild-type and isogenic mutants) were selected to constitute biofilms with varying EPS quantities due to differences in the excretion of extracellular polysaccharides. *P. aeruginosa* PAO1 (wild-type), mucA22 (excessive EPS production), and algT(U) (EPS deficient) strains were grown in one-tenth-strength Luria-Bertani (LB) broth (per liter: tryptone 1.0 g; yeast extract 0.5 g; and sodium chloride 1.0 g). *E. coli* type strain (ATCC 11775) carrying a pDsRed-Express plasmid (excitation/emission: 557 nm/579 nm; Clontech, Mountain View, CA) that produces red fluorescent protein (RFP) was chosen as a surrogate pathogen to monitor the pathogen interaction with biofilm. The *E. coli* with RFP was cultivated in 100 mL of full strength LB broth with 100 mg/mL ampicillin at 37 °C for 18-21 hours where the grown culture was then sub-cultured in a fresh medium for the same duration to promote RFP maturation.
4.2 Biofilm cultivation in a flow cell system

Initially, the *P. aeruginosa* cultures were inoculated into 200 mL of LB broth and grown overnight at 37 °C on a rotary shaker at 200 rpm. The cell pellets were harvested by centrifugation (1,620 × g, 15 minutes), re-suspended in phosphate buffer (0.54 g Na₂HPO₄ and 0.89 g KH₂PO₄ per liter), and adjusted to an optical density of 0.5 (1 × 10⁸ CFU/mL) at 600 nm. Then 2.5 mL of the *P. aeruginosa* culture was injected into a flow cell system with flow chamber dimensions (length, width, height) - 47.5 mm × 12 mm × 1.6 mm, (FC281, Biosurface Technologies, Bozeman, MT) under aseptic conditions. Cultures were retained in the flow chambers statically for four hours to initiate the bacterial attachment. Subsequently, 1/10th LB broth containing 0.005 mM of Ca²⁺ and 0.03 mM of Mg²⁺ was continuously pumped into the flow chambers at a rate of 0.2 mL/min using a multichannel cassette pump (8001-22 peristaltic pump, IDEX Health & Science, Bristol, CT) for six days to develop a confluent biofilm at room temperature (around 22°C) [13].

![Flow cell setup used for biofilm development.](image)
To investigate the influence of divalent ions on biofilm formation and subsequent pathogen interaction, the three *P. aeruginosa* biofilms were also cultivated in LB broth supplemented with divalent ions (1.7 mM of Ca$^{2+}$ and 0.9 mM of Mg$^{2+}$) representing natural waters [49] following the same flow cell experimental procedure as described before.

### 4.3 Pathogen interactions with preformed biofilms

*E. coli* with RFP was harvested by centrifugation (720 × g, 4 °C for 5 minutes) and re-suspended in phosphate buffer. The final concentration was adjusted to an absorbance of 0.5 at an optical density of 600 nm. For the interaction of the pathogen with a confluent biofilm, flow into the flow cells was temporarily suspended and 0.5 mL of the harvested *E. coli* was injected. Upon injection, flow resumed allowing the *E. coli* to flow through the flow cell chambers. To estimate the initial retention of the *E. coli*, effluent samples were collected in 10 mL serum vials every 15 minutes [1.25 × hydraulic retention time (12 min)] for up to an hour for subsequent quantification.

### 4.4 Biofilm image acquisition and analysis

The biofilm structure and *E. coli* retention in the biofilm was quantified using three-dimensional stack images taken with a multi-photon CLSM (Leica SP5) coupled with image analysis software. Prior to image acquisition, biofilms were stained with a 400 µL mixture of two fluorescent stains (0.08 mg/mL of Concanavalin A - FITC conjugate stain and 1.8 mg/mL of cell mask deep red plasma membrane stain). The
Concanavalin A stain (excitation/emission: 494 nm/521 nm, Sigma Aldrich, St. Louis, MO) was used to quantify the EPS content of biofilm by specifically targeting polysaccharide components (D-glucose and D-mannose residues) that primarily comprise *P. aeruginosa* EPS [51]. The cell mask deep red plasma membrane stain (excitation/emission: 649 nm/666 nm, Invitrogen, Grand Island, NY) targets the plasma membrane of cells to visualize *P. aeruginosa* biofilm cell biomass. The Leica LAS AF software was used to acquire z-stacked images via a 63× NA1.4 oil immersion objective. The biofilm images were collected at seven randomly selected positions on the slides. The background-noise interference in the individual images was removed using ImageJ, an image-processing program [52]. Biofilm structural characteristics including average thickness, biomass, EPS content, and surface roughness were quantitatively analyzed using a biofilm image analysis program, COMSTAT, written as a script in MATLAB 5.1 [53]. Considering the motility of *E. coli* in the biofilm, the total count of *E. coli* on each CLSM image was obtained by manual counting.

### 4.5 Quantification of *E. coli* in the effluent

To further verify pathogen retention trends in the biofilm, the quantity of *E. coli* in the effluent was enumerated by fluorescent-activated cell sorting (FACS) analysis with a FACS Calibur flow cytometer (BD Biosciences, CA). To enumerate the *E. coli*, 10 µL of a calibrated microsphere suspension (1.0 × 10^8 microspheres/mL; 6 µm in diameter; Invitrogen, Grand Island, NY) was added to 990 µL of each effluent sample, and then subjected to flow cytometry analysis [54]. WinMDI, flow cytometry analysis software, was then used to identify and quantify *E. coli* in the effluent using the obtained flow
cytometer data [55]. The \( E. coli^T \) quantity was then determined from the ratio of bacterial events to microsphere events.

4.6 Total interaction energy analysis using the DLVO theory

The size of surrogate pathogen, \( E. coli^T \), is 1-2 \( \mu \)m, thus it can be considered as a colloidal particle. As a result, the pathogen interaction with biofilm can be interpreted using classical DLVO theory, which describes the interaction energies between charged colloid particles and a surface [45]. The detailed calculations to acquire the DLVO interaction energy profile are provided below [33, 45].

The DLVO theory used to describe the net interaction energy (\( G_{TOT} \)) existing between \( E. coli^T \) and the \( P. aeruginosa \) biofilm in Luria-Bertani (LB) broth is a balance between two factors: 1) the free energy of van der Waals interaction force, \( G_{VDW} \) (general attractive); and 2) free energy of electrostatic double layer interaction force, \( G_{EDL} \) (general repulsive) [45].

\[
G_{TOT} = G_{VDW} + G_{EDL}
\]  

The van der Waals interaction force (\( G_{VDW} \)) of Eq. (1) is based on the retarded van der Waals interaction energy calculation using Gregory’s expression [56] which is defined as:

\[
G_{VDW} = -\frac{Aa}{6d} \left( \frac{1}{1 + \frac{14\pi a}{A}} \right)
\]

where \( A \) is the Hamaker constant - a material property that describes the strength of the interaction between a surface and the medium as well as between two interacting bodies in a medium, \( d \) is the separation distance between \( E. coli^T \) and \( P. aeruginosa \), \( a \) is the
radius of the cell (the cells are assumed to be spherical with 1 \(\mu\)m radius), and \(\lambda\) is the characteristic wavelength of the dielectric (assumed to be 100 nm).

The Hamaker constant \(A\) in Eq. (2) of each biofilm system was calculated from the LW (Lifshitz–van der Waals) components of the free energy of adhesion \(\Delta G_{y_0}^{LW}\) using the following equation:

\[
A = -12\pi y_0^2 \Delta G_{y_0}^{LW}
\]  
(3)

where \(y_0\) is the minimum cutoff distance between the particle and the surface which is usually assigned a value 0.157 nm [57].

\(\Delta G_{y_0}^{LW}\) of Eq. (3) is defined as:

\[
\Delta G_{y_0}^{LW} = 2 \left( \sqrt{\gamma_{i}^{LW}} - \sqrt{\gamma_{S}^{LW}} \right) \left( \sqrt{\gamma_{C}^{LW}} - \sqrt{\gamma_{i}^{LW}} \right)
\]  
(4)

where \(\gamma^{LW}\) defines as the surface free energy, thus \(\gamma_{i}^{LW}\) is the surface free energy of LB broth, \(\gamma_{S}^{LW}\) is the surface free energy of \(P.\ aeruginosa\) biofilm, and \(\gamma_{C}^{LW}\) is the surface free energy of \(E.\ coli^T\).

The surface free energies of Eq. (4) are calculated by the following equation after the contact angle was acquired using a Goniometer.

\[
(1 + \cos \theta)\gamma_{dT}^{TOT} = 2 \sqrt{\gamma_{target}^{LW} \gamma_{dT}^{LW}}
\]  
(5)

where \(\theta\) is the contact angle of diiodomethane on different bacterial surfaces; \(\gamma_{target}^{LW}\) is surface free energy of bacteria; \(\gamma_{dT}^{LW}\) is the surface free energy of diiodomethane; and \(\gamma_{dT}^{TOT}\) is the total surface energy of diiodomethane. The values of \(\gamma_{dT}^{LW}\) and \(\gamma_{dT}^{TOT}\) used were previously calculated from van Oss [57]. The surface free energy of LB broth \(\gamma_{i}^{LW}\) was acquired by measuring its contact angle on a glass slide with a known surface free energy
The electrostatic double layer interaction force \( G_{EDL} \) of Eq. (1) is based on a modified expression designed by Hogg et al. [58] for the interaction between a particle and a flat plate as following equation:

\[
G_{EDL} = \pi a \varepsilon_0 \varepsilon_r \left[ 2\psi_1 \psi_2 \times \ln \left( \frac{1 + \exp(-\kappa H_0)}{1 - \exp(-\kappa H_0)} \right) + (\psi_1^2 + \psi_2^2) \times \ln(1 - \exp(-2\kappa H_0)) \right]
\]  

(6)

where \( \varepsilon_0 \) and \( \varepsilon_r \) are the dielectric permittivity of free space in vacuum \( (8.85 \times 10^{-12} \text{ C/mV}) \) and the relative dielectric constant of solution \( (78.5 \text{ for aqueous solution}) \), \( a \) is the radius of \( E. coli^T \) \( (1 \mu\text{m}) \), \( \kappa \) is the inverse Debye length (defined as Eq. 7) [59], \( H_0 \) is the separation distance between the \( E. coli^T \) and the \( P. aeruginosa \) biofilm surface (defined as \( d \) in Eq. 2), and \( \psi_1 \) and \( \psi_2 \) are the surface potentials of the \( E. coli^T \) and \( P. aeruginosa \) biofilm surfaces measured by zetasizer, respectively.

\[
\kappa = \sqrt{\frac{2000e^2 N_A c}{\varepsilon_0 \varepsilon_r kT}}
\]

(7)

where \( e \) is an electron charge \( (1.6 \times 10^{-19} \text{ C}) \), \( N_A \) is Avogadro constant \( (6.02 \times 10^{23} \text{ mol}^{-1}) \), \( c \) is the concentration of ions in aqueous solution, \( k \) is Boltzmann constant \( (1.38 \times 10^{-23} \text{ J/K}) \), and \( T \) is the room temperature \( (298 \text{ K}) \).

As mentioned earlier, the total interaction energy between pathogen and biofilm in liquid medium is the summation of van der Waals (attractive) force and electrostatic double layer (repulsive) force, which can be determined by measuring the zeta potential and contact angles of \( E. coli^T \) and \( P. aeruginosa \) biofilm. The electrophoretic measurements of the surrogate pathogen and the bacterial biofilm were measured using a
Zetasizer (Nano – ZS90; Malvern, U.K.) and converted into surface potentials using the Smoluchowski equation (Eq. 8) [60].

$$\mu_e = \frac{\varepsilon \varepsilon_0}{\eta} \zeta$$

(8)

The bacterial cultures were prepared for measurement as follows: *E. coli* was centrifuged at a low speed (1,620 × g, 15 minutes) to minimize the stripping of EPS after cultivating in LB broth. Then, the cells were re-suspended and optical densities of the tested strains were adjusted to 0.25 at 600 nm in phosphate buffer supplemented with and without divalent ions. The *P. aeruginosa* biofilms were grown as described in Section 3.2. Upon the confluent development of biofilms, the biomass in the biofilm was carefully scraped off, re-suspended in phosphate buffer, both with and without supplementation of divalent ions, and homogenized for two minutes before measurement.

In order to calculate the van der Waals forces between the *E. coli* cell and *P. aeruginosa* biofilm, the contact angles of diiodomethane on both *E. coli* and the *P. aeruginosa* coated slides were measured in triplicate using a Goniometer (Tantec Model CAM-MICRO Contact Angle Meter; KSV Instruments Ltd., Finland). Bacterial coatings were formed by adding several drops of a highly concentrated bacterial suspension on a glass slide and then allowing the culture to dry onto the glass slide inside a laminar flow hood [33].
4.7 Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate whether a selected single parameter was significantly different among three *P. aeruginosa* strains. The pairwise comparisons using Holm-Sidak method was selected to examine the difference of each parameter between each strain.
Chapter 5

Results and discussion

5.1 *E. coli*<sup>T</sup> retention in biofilms

Acquired CLSM images and image analysis of the z-stack reveal discernible interaction between the confluent *P. aeruginosa* biofilm and the *E. coli*<sup>T</sup>. A representative image of *E. coli*<sup>T</sup> retained in the *P. aeruginosa* biofilm [algT(U)] is shown in Figure 5-1a. A large number of *E. coli*<sup>T</sup> cells were detained in the pores, channels and valleys characterizing the biofilm structure. The algT(U) biofilm possessed more empty spaces between stained biofilm cells in its structure as compared to the mucA22 and PAO1 biofilms. The *E. coli*<sup>T</sup> count results presented in Table 5.1 indicated that algT(U) detained the highest amount of *E. coli*<sup>T</sup> cells per mm<sup>2</sup> (382.1 ± 68.9) followed by mucA22 (186.1 ± 60.0) and PAO1 (82.1 ± 84.3). One-way ANOVA analysis revealed a statistically significant difference in the *E. coli*<sup>T</sup> cells retained among three *P. aeruginosa* biofilms (p=0.003). The pairwise comparison (Holm-Sidak method) results showed that algT(U) biofilm significantly detained more *E. coli*<sup>T</sup> than mucA22 and PAO1 biofilms (p < 0.05), while the difference between mucA22 and PAO1 was marginally significant (p=0.07).
Figure 5-1: Orthogonal views of a CLSM image of a six day old algT(U) biofilm after introducing *E. coli* over one hour. (a) cultivated in the LB broth (b) cultivated in the LB broth supplemented with divalent ions; Blue represents the cell biomass stained with the Cell mask deep red plasma membrane stain; green represents the EPS stained with Concanavalin A stain and red represents the *E. coli* with RFP. The circles were the highlight of *E. coli* detained in the empty spaces between the aggregated biofilm.

Table 5.1: Structural characteristics of algT(U), mucA22 and PAO1 biofilms cultivated in LB broth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>EPS content (µm³/µm²)</th>
<th>Average Thickness (µm)</th>
<th>Cell Biomass (µm³/µm²)</th>
<th>Roughness Coefficient</th>
<th>Detained <em>E. coli</em> (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>algT(U)</td>
<td>0.23 ± 0.09ᵃ</td>
<td>8.02 ± 6.14ᵃ</td>
<td>2.87 ± 1.68ᵃ</td>
<td>0.94 ± 0.50ᵃ</td>
<td>382.1 ± 68.9ᵃ</td>
</tr>
<tr>
<td>mucA22</td>
<td>0.84 ± 0.20ᵇ</td>
<td>12.19 ± 3.60ᵇ</td>
<td>6.05 ± 2.48ᵇ</td>
<td>0.60 ± 0.31ᵃ</td>
<td>186.1 ± 60.0ᵇ</td>
</tr>
<tr>
<td>PAO1</td>
<td>0.52 ± 0.32ᵃᵇ</td>
<td>9.87 ± 2.50ᵃ</td>
<td>6.89 ± 3.54ᵃ</td>
<td>0.43 ± 0.10ᵃ</td>
<td>82.1 ± 84.3ᵇ</td>
</tr>
</tbody>
</table>

Data shown as mean ± standard deviation, (n=3)

Data points with different letters in a specific parameter show statistically significant difference (p<0.05)
5.2 Enumeration of E. coli$^T$ in the effluent samples

A representative dot plot image of E. coli$^T$ from the flow cell effluent counted by FACS analysis is shown in Figure 5-2a. The E. coli$^T$ was discriminated based on the DSRED fluorescent signal. The microspheres were clearly distinguishable from the bacteria (1-2 µm) by virtue of their size (6 µm) when using the FSC (Forward Scatter) Height mode. The quantity of E. coli$^T$ in the flow cell effluent is shown in Figure 5-2b. The effluent E. coli$^T$ count was approximately $10^6 - 10^7$ CFU/mL for all three P. aeruginosa biofilms. The results indicated that the majority of E. coli$^T$ cells were temporarily detained in the flow cell system, but were subsequently detached and flushed from the flow cell system. Figure 5-2b indicates slightly higher detachment of E. coli$^T$ cells from the PAO1 biofilms than the mucA22 and algT(U) biofilms. A considerable correlation was observed between the E. coli$^T$ count in the effluent by FACS analysis and the number of E. coli$^T$ cells detained in the biofilms determined by the CLSM analysis. Higher entrapment of E. coli$^T$ was observed in the algT(U) and mucA22 biofilms leading to a lower number of E. coli$^T$ cells released in the effluent. PAO1 biofilm detained a lower fraction of E. coli$^T$ leading to an increase in E. coli$^T$ release from the flow cell effluent (Figure. 5.2b). The FACS analysis results verified the differences observed for the E. coli$^T$ detention in the biofilm acquired via CLSM image analysis.

As per literature, E. coli when grown in the presence of LB broth has been reported to have a doubling time ($\tau$) = 20-30 minutes [61, 62]. Since the retention time of the inoculated bacterial cultures into the flow cells were 12.5 minutes and our data indicates that the samples were collected every 15 minutes, we strongly believe a significant number of E. coli$^T$ that was injected into the flow cells would get detached from the flow
cell in the first 15 minutes considering the doubling time of \( E. \ coli^{T} \). Hence, the effect of growth and multiplication of \( E. \ coli^{T} \) within the flow cells can be neglected as most of the injected \( E. \ coli^{T} \) gets detached even before they double.

**Figure 5-2:** FACS analysis of \( E. \ coli^{T} \) retention in biofilms. (a) A plot representing the number of events vs DSRED (Left) and a reconstructed, re-gated plot using winMDI for \( E. \ coli^{T} \) enumeration (Right); (b) Effluent \( E. \ coli^{T} \) cell number from the three phenotypic variant (algT(U), mucA22 and PAO1) biofilms grown without divalent ions at 15 minutes intervals.

### 5.3 EPS influence on biofilm structural characteristics

To understand differences in \( E. \ coli^{T} \) detention among the three \( P. \ aeruginosa \) biofilms, structural characteristics of biofilm were determined and compared using COMSTAT on the stacked CLSM biofilm images (Table 5.1). The determination of biofilm EPS content from individual strains was consistent with its EPS excretion capacity. The algT(U) biofilm produced the least EPS content \((0.23 \pm 0.09 \, \mu m^3/\mu m^2)\), while the PAO1 biofilm produced a modest EPS content \((0.52 \pm 0.32 \, \mu m^3/\mu m^2)\) and the mucA biofilm had the highest EPS content \((0.84 \pm 0.20 \, \mu m^3/\mu m^2)\). A pairwise comparison revealed that the EPS content of mucA22 was significantly higher than the algT(U) \((p<0.05)\).
Although some studies have reported that increased biofilm EPS content might facilitate the retention of pathogenic bacteria \([37, 45]\), there was no direct correlation found between EPS content and the detained \textit{E. coli}\textsuperscript{T} quantities. To further understand whether other biofilm parameters account for the \textit{E. coli}\textsuperscript{T} detention, structural characteristics of biofilms (the average thickness, cell biomass, and roughness coefficient) were determined and compared. First, algT(U) strain had the thinnest average biofilm thickness (8 µm), while the average thickness of mucA22 and PAO1 biofilms were 12 µm and 10 µm, respectively. This trend appears to be correlated to the EPS content of the biofilm. Biofilm containing minimal EPS has been reported to produce thinner biofilms, because the absence of EPS inhibits development of mature biofilm with microcolony structures \([39, 63]\). Biofilm producing more EPS can form a thicker microcolony structure that would increase EPS filled spaces between microbial cells in the biofilm; thus, mucA22 was found to have the highest average biofilm thickness among three, while algT(U) was observed to have the lowest average thickness due to its EPS deficiency.

Second, PAO1 biofilms had 10% more biomass (6.05 ± 2.48 µm\(^3/\mu m^2\)) than mucA22 biofilms and 60% more biomass than algT(U) biofilms. The cell biomass content represents the overall volume of the cells presented as the ratio of biomass volume to the biofilm substratum-area. The minimal value observed for algT(U) could be attributed to its diminished EPS production capacity that inhibits development of microcolony structures in the biofilm. The last biofilm parameter examined was the surface roughness of the biofilm, which is a measure of biofilm thickness variation indicating the biofilm structural heterogeneity characterized by the presence of empty
spaces and irregular structure between biofilm cells. Surface roughness \( R_a^* \) can be defined by the following equation as suggested by Heydorn et al. 2000 [53]:

\[
R_a^* = \frac{1}{N} \sum_{i=1}^{N} \frac{|L_{fi} - L_f|}{L_f}
\]

where \( L_{fi} \) = ith individual thickness measurement, \( L_f \) = mean thickness, \( N \) = number of thickness measurements.

In this study, algT(U) biofilms exhibited a higher surface roughness value (0.94 ± 0.50) than the mucA22 (0.60 ± 0.31) and PA01 biofilms (0.43 ± 0.10). The algT(U) biofilms possessed a non-uniform, discontinuous, and greatly heterogeneous structure, contributing to higher surface roughness values over the other biofilms.

5.4 *E. coli* retention in biofilms in the presence of divalent ions

To better simulate the pathogenic bacteria interaction with biofilm in the environment, experiments were repeated using LB broth supplemented with a divalent ion concentration typically found in freshwater aquatic environments. The CLSM images showed that biofilms grown with divalent ion supplementation had scattered patch-like structures with low EPS content (Figure 5-1b). Some biofilms had notably pillared structures that created localized areas with elevated thickness. This loose and scattered biofilm structure was also observed in a *Paracoccus sp.* biofilm cultivated with divalent ions [64].

Despite structural differences, significant increases in *E. coli* retention were observed for all three biofilms with divalent ion supplementation. Although large variance was presented due to the heterogeneity of biofilms, overall *E. coli* retention
trend showed that the algT(U) biofilm still detained a higher number of *E. coli* cells (562.51 ± 333.48) than those of mucA22 (442.56 ± 259.87) and PAO1 (503.95 ± 298.58) biofilms presented in Table 5.2.

**Table 5.2: Structural characteristics of algT(U), mucA22 and PAO1 biofilms cultivated in the LB broth supplemented with divalent ions.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>EPS content (µm³/µm²)</th>
<th>Average Thickness (µm)</th>
<th>Cell Biomass (µm³/µm²)</th>
<th>Roughness Coefficient</th>
<th>Detained <em>E. coli</em> T (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>algT(U)</td>
<td>0.12 ± 0.12a</td>
<td>6.70 ± 9.54a</td>
<td>1.02 ± 0.88a</td>
<td>1.58 ± 0.34a</td>
<td>562.51 ± 333.48a</td>
</tr>
<tr>
<td>mucA22</td>
<td>0.18 ± 0.17a</td>
<td>18.90 ± 13.88a</td>
<td>3.09 ± 1.48a</td>
<td>0.98 ± 0.29a</td>
<td>442.56 ± 259.87a</td>
</tr>
<tr>
<td>PAO1</td>
<td>0.18 ± 0.01a</td>
<td>3.82 ± 3.31a</td>
<td>1.37 ± 0.88a</td>
<td>1.22 ± 0.41a</td>
<td>503.95 ± 298.58a</td>
</tr>
</tbody>
</table>

Data shown as mean ± standard deviation, (n=3)
Data points with different letters in a specific parameter show a statistically significant difference (p<0.05)

5.5 The influence of divalent ions on biofilm structure

Table 5.2 lists the biofilm structural characteristics of the biofilms cultivated under the influence of divalent ions. Drastic difference in the biofilm structures were observed with the supplementation of divalent ions. The biofilms had fluffy, uneven, tall, pillar like structure with large voids and cavities between the micro colony structures.

Given the noticeable differences in biofilm structure for all strains under divalent ion supplementation, the addition of Ca²⁺ and Mg²⁺ to LB broth could cause an unexpected malnutrition condition, phosphate starvation, in bacteria, which led to the higher variance for all measured structural parameters of biofilm. The solubility product constant (*K*ₚₛ) at 25 °C for calcium phosphate and magnesium phosphate are 2.07 × 10⁻³³ and 1.04 × 10⁻³³ which indicates these two compounds are highly insoluble. With the addition of Ca²⁺ and Mg²⁺ into LB broth, phosphate would be stripped from the broth.
forming insoluble \( \text{Ca}_3(\text{PO}_4)_2(\text{S}) \) and \( \text{Mg}_3(\text{PO}_4)_2(\text{S}) \). The growth of \( P. \text{aeruginosa} \) biofilm considering phosphate deficiency could induce the formation of reactive oxygen species inside the cell and limit bacterial growth [65].

The phosphate malnutrition induced by the supplementation of divalent ions manifested its effect on several biofilm structural parameters. For instance, the EPS content (\( \mu m^3/\mu m^2 \)) of \( \text{algT(U)} \), \( \text{mucA22} \) and \( \text{PAO1} \) biofilms were \( 0.12 \pm 0.12, 0.18 \pm 0.17 \) and \( 0.18 \pm 0.01 \), respectively. While the \( \text{algT(U)} \) EPS content remained the lowest among strains, the EPS contents for all three strains were lower than their corresponding biofilms cultivated without divalent ion supplementation. This condition could be caused by the phosphate starvation, forcing the bacteria in the biofilm to limit the production of EPS. Secondly, the average thickness of the \( \text{algT(U)} \) and \( \text{PAO1} \) biofilms was reduced to 7 \( \mu m \) and 4 \( \mu m \), respectively, while the average thickness of \( \text{mucA22} \) increased from 12 \( \mu m \) to 19 \( \mu m \). Average thickness values of the individual biofilms reduced even though there was an increase in the maximum thickness of the biofilms (data not presented) due to the tall pillar like structures. The probable reason that can be attributed to the decreased average thickness of the biofilms could be due to the irregular structure of the biofilms. Sarkisova et al. reported a similar observation with the increased average thickness of a mucoid strain \( P. \text{aeruginosa} \) under the influence of calcium ions [23]. Calcium ions were reported to increase the biofilm thickness by cross-linking the uronic acid residuals in EPS, as \( \text{mucA22} \) is genetically induced to overproduce EPS. Moreover, when grown with divalent ions, \( \text{mucA22} \) biofilms had 56% more biomass than \( \text{PAO1} \) biofilms and 67% more biomass content than \( \text{algT(U)} \) biofilms. The highest cell biomass content of mucoid strain (\( \text{mucA22} \)) among the three biofilms might also be related to
cross-linked EPS effects induced by calcium ions [23]. Finally, all three strains were shown to have higher heterogeneity (roughness coefficient) when cultivated with divalent ion supplementation. The phosphate starvation appeared to limit the growth of confluent biofilm, contributing to large empty spaces between patch-like biofilm structures, which significantly increased the biofilm heterogeneity.

As mentioned earlier, Kuznar et al. [18] observed a surface charge neutralization condition under the influence of Ca\(^{2+}\) ions. Kuznar et al.’s results were clearly evidenced by the reduced zeta potential values that contributed to the reduction of the repulsive energy barrier between the pathogen and the substratum surface. Even though a reduction of zeta potential values was observed under the influence of divalent ions in our study (Table 5.5), the difference in the zeta potential values was however not significant among the three cultures. However, there was a reduction in the repulsive energy barrier (Table 5.6) between the individual biofilms and the pathogenic microorganism (E. coli\(^T\)).

Calcium ions are well known to facilitate bacterial association by forming cationic bridges between the polymeric molecules in the individual bacterial cells [20]. Several authors have reported that divalent ions stimulate the development of thick, continuous, stable biofilm structures [21-23]. However in our experiments, we observed tall, pillar-like, discontinuous biofilm structures under the influence of divalent ions that was completely contradicting to that observed by other authors. Hence, bridging effect cannot be the mechanism responsible for the increase in retention of E. coli\(^T\) by the biofilms under the influence of divalent ions. Hence, based on our results it was clear that another mechanism other than the surface charge neutralization, electrical double layer
compression, and bridging effect was involved in the increased retention of pathogens within the biofilms.

5.6 Correlation between biofilm structural parameters and $E. coli^T$ retention

To investigate whether biofilm structural characteristics were directly correlated to the $E. coli^T$ detention in the biofilms; the structural characteristics of biofilms cultivated with and without divalent ion supplementations were combined and correlated to the detained $E. coli^T$ quantities. Among all observed biofilm structural parameters, the surface roughness coefficient was the only parameter that significantly correlated to the detained $E. coli^T$ quantity ($R^2 = 0.92$, Figure 5-3), where a higher surface roughness coefficient value corresponded to increased $E. coli^T$ detention in the biofilm. The surface roughness of biofilm was previously shown to increase the bacterial attachment to biofilms in the river systems and on nanofiltration membranes [63, 66]. The void regions between bacterial biofilm micro colonies create a quiescent zone without any fluid flow; thus, the $E. coli^T$ entering into the void region can be temporarily detained in the biofilm where subsequent interactions may facilitate more permanent attachment. The remaining structural characteristics of biofilm did not have any notable correlation to the amount of detained $E. coli^T$. 
Figure 5-3: The quantity of \( E. \ coli^T \) detached versus surface roughness coefficient. Closed symbols represent the roughness values of biofilms cultivated without divalent ion supplementation and the open symbols represent the biofilms cultivated with divalent ion supplementation.

5.7 Total interaction energy analysis between \( E. \ coli^T \) and \( P. \ aeruginosa \) biofilm

Beyond the physical structure of the biofilm, however, interaction energy between the pathogen and the biofilm may affect pathogen retention in biofilm. Accordingly, the mechanism behind the retention was further investigated by considering total interaction energy between the pathogen and biofilm in liquid medium, which is interpreted using the DLVO theory. The DLVO theory has been applied to interpret bacterial adhesion on homogeneous surfaces such as quartz and glass [28, 45, 49], but the interpretation between biofilm and bacteria interaction has not been well explored [45].

The van der Waals interactions, the attractive force of DLVO theory, were acquired by measuring the contact angles of \( P. \ aeruginosa \) biofilms, planktonic \( E. \ coli^T \), and LB broth (Table 5.3), followed by calculating the surface free energy and the unique Hamaker constant for each strain. The Hamaker constants, that can determine the van der
Waals interaction energy among three \( P. \ aeruginosa \) biofilm systems (PAO1, mucA22, and algT(U)), were \( 4.84 \times 10^{-22} \) Joules (J), \( 1.12 \times 10^{-21} \) J, and \( 2.79 \times 10^{-21} \) J, respectively as shown in Table 4.4. The highest Hamaker constant was found for algT(U) biofilm which contributed to the highest van der Waals attractive energy among the three tested biofilm systems. Additionally, the electrostatic double layer interaction, the repulsive force of the DLVO theory, was acquired by measuring the zeta-potential of the three \( P. \ aeruginosa \) biofilms and planktonic \( E. \ coli \) in the LB broth with and without divalent ion supplementations (Table 5.5).

Table 5.3: Contact angle and surface free energy of tested bacteria and medium.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Contact angle ( \theta ) (g2237)</th>
<th>( \gamma_{LW} ) (mJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB broth</td>
<td>--</td>
<td>47.35</td>
</tr>
<tr>
<td>( E. \ coli )</td>
<td>52.33 ± 2.52</td>
<td>32.98</td>
</tr>
<tr>
<td>( P. \ aeruginosa ) PAO1</td>
<td>30.00 ± 2.00</td>
<td>16.92</td>
</tr>
<tr>
<td>( P. \ aeruginosa ) mucA22</td>
<td>38.66 ± 1.15</td>
<td>31.20</td>
</tr>
<tr>
<td>( P. \ aeruginosa ) algT(U)</td>
<td>56.00 ± 2.64</td>
<td>43.62</td>
</tr>
</tbody>
</table>

Table 5.4: Free energy of van der Waals component (\( \Delta G_{LW}^{B_0} \)) and Hamaker constant (A) of tested biofilm systems.

<table>
<thead>
<tr>
<th>Biofilm system</th>
<th>( \Delta G_{LW}^{B_0} ) (mJ)</th>
<th>( A ) (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P. \ aeruginosa ) PAO1</td>
<td>-0.52</td>
<td>( 4.84 \times 10^{-22} )</td>
</tr>
<tr>
<td>( P. \ aeruginosa ) mucA22</td>
<td>-1.21</td>
<td>( 1.12 \times 10^{-21} )</td>
</tr>
<tr>
<td>( P. \ aeruginosa ) algT(U)</td>
<td>-3.01</td>
<td>( 2.79 \times 10^{-21} )</td>
</tr>
</tbody>
</table>
Table 5.5: Surface potentials of the bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Surface potential cultivated in the LB broth (mV)</th>
<th>Surface potential cultivated in the LB broth supplemented with divalent ions (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planktonic cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> T</td>
<td>-47.66 ± 1.89</td>
<td>-18.33 ± 0.50</td>
</tr>
<tr>
<td>Biofilm cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>-21.70 ± 0.40</td>
<td>-21.63 ± 1.30</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> mucA22</td>
<td>-32.66 ± 0.11</td>
<td>-27.20 ± 1.12</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> algT(U)</td>
<td>-31.83 ± 0.56</td>
<td>-26.90 ± 0.72</td>
</tr>
</tbody>
</table>

The total interaction energy between *E. coli* T and *P. aeruginosa* biofilm in the LB broth is the sum of the van der Waals and electrostatic double layer interaction energies. Figure 5-4a shows the total interaction energies for the biofilm systems. As illustrated in Figure 5-4a, the calculation predicted that *E. coli* T was attracted to the *P. aeruginosa* biofilm at a separation distance of 9.2-17.6 nm as indicated by a negative interaction energy dominated by van der Waals attractive interaction. The lowest interaction energy and corresponding separation distance are termed as secondary minimum depth and secondary minimum separation distance, respectively [49]. Figure 5-4b emphasizes the magnitude of this transition area. When the distance between *E. coli* T and *P. aeruginosa* biofilm is compressed, the influence of the electrostatic double layer interaction becomes significant, causing the total interaction energy to abruptly enter the repulsive region (positive interaction energy). However, the van der Waals attractive energy also increased when the separation distance decreases, which reduces the total interaction energy between *E. coli* T and *P. aeruginosa* biofilms.
Figure 5-4: Total interaction energy profiles between the $E. coli^T$ and the three $P. aeruginosa$ biofilms under different divalent ion concentrations. (a) Total interaction energy profile; (b) The amplified region emphasizing the secondary minimum depth and separation distance.

The total interaction energy decreases when the rate of van der Waals attractive energy increases in excess of the electrostatic double layer repulsive energy, where the highest point in the energy curve is termed as the energy barrier. The values of the energy barrier, secondary minimum depth, and secondary minimum separation distance are presented in Table 5.6 indicates that all tested biofilm systems had attractive secondary minimum separation energy, suggesting that $E. coli^T$ could undergo reversible attachment to $P. aeruginosa$ biofilm surface. However, permanent attachment of $E. coli^T$ requires that a considerable energy barrier (701 to 1740 kT) must be overcome.
Table 5.6: The profile of the DLVO parameters for interaction between three *P. aeruginosa* biofilms and *E. coli* in two different divalent concentrations.

<table>
<thead>
<tr>
<th>Strains of <em>P. aeruginosa</em></th>
<th>Energy barrier (kT)</th>
<th>Secondary minimum depth (kT)</th>
<th>Secondary minimum separation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB broth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>algT(U)</td>
<td>753</td>
<td>-2.4</td>
<td>13.2</td>
</tr>
<tr>
<td>mucA22</td>
<td>1710</td>
<td>-0.7</td>
<td>16.1</td>
</tr>
<tr>
<td>PAO1</td>
<td>1740</td>
<td>-0.3</td>
<td>17.6</td>
</tr>
<tr>
<td>LB broth supplemented with divalent ions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>algT(U)</td>
<td>701</td>
<td>-4.3</td>
<td>9.2</td>
</tr>
<tr>
<td>mucA22</td>
<td>1232</td>
<td>-1.3</td>
<td>11.2</td>
</tr>
<tr>
<td>PAO1</td>
<td>1296</td>
<td>-0.4</td>
<td>14.8</td>
</tr>
</tbody>
</table>

The obtained experimental results indicate that *E. coli* was retained in the biofilm beyond transient interaction. Previous studies reported that the bacterial surface structure, such as the physical appendages of bacteria (flagella, fimbriae, and pili); facilitate bacterial attachment by overcoming the repulsive energy barrier [16, 46, 67]. Another possible alternative explanation states hydroxyl groups on the biofilm surface form hydrogen bonds with the hydroxyl groups on the pathogen surface [68]. Each hydrogen bond is approximately 2.5 kT; therefore, several hundred hydrogen bonds can generate a force sufficient to bridge the pathogen and the surface of the biofilm [37].

When comparing the DLVO analysis profile among the biofilm systems of three strains to predict the retention of *E. coli*, the algT(U) biofilm would attract the most *E. coli* as its energy barrier was smallest, while the PAO1 biofilm would attract the fewest of *E. coli* due to its highest energy barrier (Table 5.6). This trend is consistent with
experimental results of retained *E. coli*<sup>T</sup> as obtained by CLSM image analysis (Tables 5.1 and 5.2).

The divalent ion influence prediction from the DLVO theory revealed that the biofilm systems cultivated with divalent ion supplementations would detain more *E. coli*<sup>T</sup> than the non-supplemented. This increased retention would be caused by a decrease in repulsive energy as indicated by reduced zeta potential of the both the *E. coli*<sup>T</sup> and the biofilm surface in the presence of divalent ions. These predictions using the DLVO theory are consistent with the observed *E. coli*<sup>T</sup> retention in the biofilm systems. Among the three parameters (energy barrier, secondary minimum depth, and secondary minimum separation distance) calculated by the DLVO theory, the secondary minimum separation distance was the only parameter possessing a conceptually reasonable correlation to the *E. coli*<sup>T</sup> detention, (R<sup>2</sup> = 0.71).
Chapter 6

Conclusions

This study investigated how biofilm structure and divalent ions influence pathogen retention in biofilm after observing the short-term interaction between *E. coli* and preformed *P. aeruginosa* biofilms which possess different structural characteristics due to variable EPS production capacities. The results clearly indicated the heterogeneity of biofilm could create a quiescent zone that aided the temporary retention of *E. coli* in biofilms. The presence of divalent ions in the solution not only greatly enhanced the pathogen retention, but also greatly increased the heterogeneity of biofilm structure.

The interpretation of obtained results using the DLVO theory further indicated the surface characteristics of the biofilm induced by EPS production influenced the magnitude of the energy barrier that must be overcome for initial pathogen attachment. The DLVO analysis also showed the increased divalent ion concentration had even greater impact on energy barrier reduction to facilitate pathogen retention. Although these results provide adequate information to describe the initial attachment of pathogen to biofilm, they cannot predict the long-term pathogen retention and proliferation in biofilm. Overall, this study provides insight toward understanding the retention of the pathogenic bacteria in biofilms.
Chapter 7

Recommendations for future research

This chapter provides a list of topics for future studies recommended based upon our work.

a) To understand the harborage and incorporation of a pathogen into the biofilm, a longer duration experimental study (15 days) can be carried out.

b) *E. coli*\(^T\) was used as a pathogen in this study to check its interaction with preformed biofilms. Other organisms like *Bacillus* spores commonly used as surrogates in anthrax studies can be used to check the difference in their interaction with preformed biofilm when compared to *E. coli*\(^T\).

c) A quorum sensing study can be carried out to provide a detailed insight into the community response mechanism of pathogen interactions with preformed biofilm.

d) We believe all biofilms do not interact the same way with pathogenic microorganisms. In our study, the *P. aeruginosa* biofilms developed were carbohydrate based biofilms and in the future *P. putida* biofilms can be
considered as they are protein based biofilms. The results can be compared to check if the protein and carbohydrate based biofilms interact the same way.
REFERENCES


