Interactions between Biofilm Growth and Fluid Flow in Porous Media

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Interactions between Biofilm Growth and Fluid Flow in Porous Media

by

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A THESIS

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Abstract

Many engineered and natural systems are influenced by biofilms, which are surface attached communities of microorganisms embedded in a matrix composed of microbially produced extracellular polymeric substances. In porous media, biofilms can progressively accumulate within pore spaces, making it increasingly difficult for fluids to flow through the pore network. Therefore, biofilms are often considered to be detrimental in processes relying on fluid flow in porous media. Engineering biofilm development in porous structures, however, can maximize the beneficial aspects of biofilms while minimizing their detrimental effects. Due to an inadequate understanding of interactions between flow properties and biofilm development, field scale applications of biofilm based processes are still unpredictable.

This study was divided to two main parts. The first part focused on a theoretical investigation of biofilm growth in porous media. By treating biofilm as an evolving viscous fluid that shares void spaces with a separate aqueous phase in porous media, a novel macroscopic approach was developed to simulate biofilm growth in porous media. Modelling results revealed that relative permeability functions can be used to link flow of water to biofilm saturation. However, this modelling approach was complex, and certain model parameters needed to be experimentally determined.

Therefore, in the second part of the study, by using a bioluminescent bacterium, a noninvasive imaging method was developed to visualize biofilm evolution within porous media. Detected bioluminescence intensities were used to nondestructively quantify biofilm and porous media characteristics. The imaging technique was also
used to study bacterial transport in porous media with different hydraulic properties. Results indicated that biofilm formation can significantly improve bacterial sticking efficiency in porous media by modifying hydrophobicity of solid surfaces. Finally, the developed imaging technique was used to monitor biofilm development under a constant pressure gradient in a two-dimensional flow field. Results revealed that in porous media with small pore sizes and low permeabilities, biofilm grows predominantly in upstream regions toward the nutrient source and against the fluid flow, whereas in porous media with coarse pore sizes and elevated permeabilities, biofilm primarily disperses in the downstream direction, in the same direction as the fluid flow, but away from the nutrient source. Observed differences in growth patterns could be explained by considering the pore size distribution in a given medium.
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# Table of Contents

Approval Page ............................................................................................................................... i  
Abstract ........................................................................................................................................ ii  
Acknowledgments .................................................................................................................... iv  
Table of Contents ....................................................................................................................... vi  
List of Tables ............................................................................................................................... xi  
List of Figures ............................................................................................................................. xii  
Nomenclature .............................................................................................................................. xxiii  

## Chapter 1
Introduction .................................................................................................................................. 1  
1.1 Motivation and Goals ....................................................................................................... 1  
1.2 Problem Statement ........................................................................................................... 6  
1.3 Thesis Overview .............................................................................................................. 8  

## Chapter 2
Literature Review ...................................................................................................................... 11  
2.1 Overview ....................................................................................................................... 11  
2.2 Porous Media .................................................................................................................. 12  
  2.2.1 Porosity .................................................................................................................. 13  
  2.2.2 Phase Saturation ................................................................................................. 13  
  2.2.3 Absolute Permeability ......................................................................................... 13  
  2.2.4 Darcy Equation ................................................................................................. 14  
2.3 Bacteria ........................................................................................................................... 16  
2.4 Biofilms – advanced microbial communities ................................................................... 19  
  2.4.1 Biofilm Formation .............................................................................................. 23  
  2.4.2 Biofilm Composition ......................................................................................... 25  
  2.4.3 Biofilm Matrix ................................................................................................. 26  
2.5 Bioclogging ..................................................................................................................... 29  
2.6 Approaches and Techniques for Studying Porous Media Biofilms .................................. 33  
  2.6.1 Biofilm Imaging in Porous Media .................................................................. 34  
  2.6.2 Porous Media Flow Chambers in Biofilm Research ..................................... 39  
2.7 Importance and Applications of Biofilms ......................................................................... 40  

## Chapter 3
A New Approach to Model the Spatiotemporal Development of Biofilm Phase in Porous Media ......................................................................................................................... 47  
3.1 Preface ............................................................................................................................ 47
3.2 Abstract ............................................................................................................. 48
3.3 Introduction ....................................................................................................... 49
3.4 Background ....................................................................................................... 51
3.5 Materials and Methods ...................................................................................... 54
  3.5.1 Model Formulation and Implementation ................................................... 54
  3.5.2 Fluid Flow and Component Transport ....................................................... 55
  3.5.3 Reactions .................................................................................................... 59
  3.5.4 Numerical Model ....................................................................................... 62
  3.5.5 Model Validation: Two-Dimensional (2D) Experiments ......................... 63
    3.5.5.1 Partially Inoculated Sandbox .............................................................. 63
    3.5.5.2 Fully Inoculated Sandbox ................................................................... 65
3.6 Results and Discussion ..................................................................................... 67
  3.6.1 Comparison of New Model versus Experiments ....................................... 67
    3.6.1.1 Partially inoculated sandbox ............................................................... 69
    3.6.1.2 Fully inoculated sandbox .................................................................... 76
  3.6.2 Effects of Relative Permeability Curves .................................................... 79
3.7 Conclusions ....................................................................................................... 82

Chapter 4
Real Time Monitoring of Biofilm Development Under Flow Conditions in Porous Media ........................................................................................................................... 83
  4.1 Preface .............................................................................................................. 83
  4.2 Abstract ............................................................................................................. 84
  4.3 Introduction ....................................................................................................... 85
  4.4 Background ....................................................................................................... 86
  4.5 Materials and Methods ...................................................................................... 88
    4.5.1 Bacterial Strain ........................................................................................... 88
    4.5.2 Media ......................................................................................................... 88
      4.5.2.1 Stock Culture Growth Medium ............................................................ 89
      4.5.2.2 Medium for Biofilm Growth in Flow Chamber ................................... 89
      4.5.2.3 Induction Medium ................................................................................ 90
    4.5.3 Chamber Inoculation .................................................................................. 90
    4.5.4 Setup and Operation of the Flow Chamber ................................................ 91
    4.5.5 Bioluminescence Imaging .......................................................................... 95
    4.5.6 Image Processing ....................................................................................... 96
      4.5.6.1 Assignment of Bioluminescence Intensity Values ............................. 96
      4.5.6.2 Image Segmentation ............................................................................ 98
    4.5.7 Additional Measurements ........................................................................ 100
      4.5.7.1 Dissolved Oxygen Determination ...................................................... 100
      4.5.7.2 Quantification of Bacterial Cell Populations ................................. 101
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5.7.3</td>
<td>Protein Measurement</td>
<td>102</td>
</tr>
<tr>
<td>4.5.7.4</td>
<td>Direct Visualization</td>
<td>102</td>
</tr>
<tr>
<td>4.6</td>
<td>Results and Discussions</td>
<td>103</td>
</tr>
<tr>
<td>4.6.1</td>
<td>Spatiotemporal Development of Biofilm in Porous Medium</td>
<td>103</td>
</tr>
<tr>
<td>4.6.2</td>
<td>Two-Way Induction</td>
<td>107</td>
</tr>
<tr>
<td>4.6.3</td>
<td>Analysis of Bioluminescence Images</td>
<td>110</td>
</tr>
<tr>
<td>4.6.4</td>
<td>Biological Analyses</td>
<td>111</td>
</tr>
<tr>
<td>4.6.5</td>
<td>Porous Medium Hydraulic Properties</td>
<td>115</td>
</tr>
<tr>
<td>4.7</td>
<td>Conclusions</td>
<td>119</td>
</tr>
</tbody>
</table>

### Chapter 5

Application of Bacterial Bioluminescence in Porous Media to Evaluate Biofilm Characteristics and Hydrodynamic Properties | 120 |
---|---|
5.1 | Preface | 120 |
5.2 | Abstract | 121 |
5.3 | Introduction | 122 |
5.4 | Materials and Methods | 126 |
| 5.4.1 | Bacterial strain | 126 |
| 5.4.2 | Culture Media | 126 |
| 5.4.3 | Porous Media | 127 |
| 5.4.4 | Dissolved Oxygen Measurement | 128 |
| 5.4.5 | Imaging | 128 |
| 5.4.6 | Experimental Procedures | 129 |
| 5.4.6.1 | Effect of Oxygen on Bacterial Bioluminescence | 129 |
| 5.4.6.2 | Bioluminescence of Bacteria in Planktonic Form | 129 |
| 5.4.6.3 | Bioluminescence of Bacteria in Biofilm Form | 131 |
| 5.4.7 | Gravimetric Measurements | 133 |
| 5.4.8 | Bacterial Cell Enumeration | 135 |
5.5 | Results and Discussions | 136 |
| 5.5.1 | Oxygen Effects | 136 |
| 5.5.2 | Cell Density Evaluation | 139 |
| 5.5.3 | Biofilm Saturation | 142 |
| 5.5.4 | Hydraulic Conductivity | 143 |
5.6 | Conclusion | 147 |

### Chapter 6

Effect of Extracellular Polymeric Substances on Bacterial Fate and Transport in Porous Media | 148 |
---|---|
6.1 | Preface | 148 |
6.2 | Abstract | 149 |
6.3 Introduction .................................................................................................................. 150
6.4 Materials and Methods ............................................................................................. 155
  6.4.1 Bacterial strain ..................................................................................................... 155
  6.4.2 Culture Preparation ......................................................................................... 155
  6.4.3 Porous Media ................................................................................................... 157
  6.4.4 Column Experiments ....................................................................................... 158
  6.4.5 Bioluminescence Imaging ................................................................................. 161
  6.4.6 Bacterial Enumeration ..................................................................................... 161
  6.4.7 Model Development .......................................................................................... 162
6.5 Results and Discussion ............................................................................................ 166
  6.5.1 Parameter Estimation ...................................................................................... 166
  6.5.2 Effect of Collector Size on Bacterial Removal Efficiency .................................. 167
  6.5.3 Influence of Biofilm on Bacterial Attachment .................................................. 171
6.6 Conclusions .............................................................................................................. 175

Chapter 7
Effects of Porous Medium Hydraulic Properties on Biofilm Growth Patterns in Porous Media .......................................................................................................................... 177
  7.1 Preface ................................................................................................................... 177
  7.2 Abstract .................................................................................................................. 178
  7.3 Introduction ............................................................................................................ 179
  7.4 Materials and Methods .......................................................................................... 185
    7.4.1 Microorganism ................................................................................................. 185
    7.4.2 Growth and Induction Media ......................................................................... 185
    7.4.3 Inoculum Preparation ...................................................................................... 187
    7.4.4 Porous Media .................................................................................................. 187
    7.4.5 Flow Chamber .................................................................................................. 189
    7.4.6 Chamber Sterilization ..................................................................................... 192
    7.4.7 Experimental Procedures ................................................................................ 193
      7.4.7.1 Operation of Flow Chamber ...................................................................... 193
      7.4.7.2 Bioluminescence Imaging ......................................................................... 194
      7.4.7.3 Bacterial Cell Enumeration ....................................................................... 197
      7.4.7.4 Protein Measurement ................................................................................ 198
  7.5 Results and Discussion ............................................................................................ 199
    7.5.1 Hydraulic Variations ....................................................................................... 199
    7.5.2 Spatial Development of Biofilm ...................................................................... 203
    7.5.3 Biological Analyses ......................................................................................... 205
    7.5.4 Tracer Experiments .......................................................................................... 209
  7.6 Conclusions .............................................................................................................. 215
List of Tables

Table 2-1: Summary of typical bacterial cell structures .............................................. 18
Table 2-2: General composition of biofilms (Sutherland, 2001) ................................. 26
Table 2-3: EPS related functions in bacterial biofilms (adapted from Wingender et al., 1999) .................................................................................................... 28
Table 2-4: Bioclogging results in different experiments indicating the influence of biofilm growth on porous media permeability reduction. Results indicate maximum permeability reductions achieved in bioclogging experiments ................................................................................................ 31
Table 3-1: Properties of the partially inoculated sandbox experiment (Kildsgaard and Engesgaard, 2002) ............................................................................... 64
Table 3-2: Properties of the fully inoculated sandbox experiment (Thullner et al., 2004) .......................................................................................................... 66
Table 3-3: Parameters used for the water and biofilm phase relative permeability functions (Equation 3.6 and 3.7) in order to simulate 2D biofilm experiments ............................................................................................................. 68
Table 3-4: Dimensionless parameters used in relative permeability functions (Equations 3.6 and 3.7) to simulate and investigate the effect of relative permeabilities on the overall biofilm phase development ......................... 79
Table 6-1: Experimental conditions for breakthrough experiments and parameters used to study bacterial attachment in porous media .............................. 157
Table 6-2: Dimensionless numbers used to calculate single collector collision efficiency ............................................................................................................. 164
Table 6-3: Calculated hydraulic properties of clean packed beds .............................. 166
Table 6-4: Bacterial concentrations in column effluents and determined attachment coefficients for each packed bed ................................................................. 168
Table 7-1: Characteristics of 3 different porous media used to study biofilm spatiotemporal development ................................................................. 188
Table A-1: Composition of solutions used to prepare growth medium ................. 247
List of Figures

Figure 1-1: Illustration of development of biofilm in porous media alters the hydraulic properties of porous structure. Increased amount of biofilm reduces the available pore space to fluid flow and may plug the pore throats, resulting in lower permeability (i.e. bioclogging) ...........................2

Figure 1-2: Bioclogging due to biofilm growth on solid particles in a porous medium (only 5 particles displayed) which can either reduce the available pore space to fluid flow or plugging the pore throats ..........3

Figure 2-1: Porous media, a solid matrix with interconnected network of voids (pores) filled with a fluid ......................................................................................................................12

Figure 2-2: Cutaway sketch of a typical bacterial cell showing structural compartments .................................................................................................................................17

Figure 2-3: Bacterial biofilm formed on a piece of glass slide. Bacterial cells permanently attach to the solid surface by secretion of EPS in which they are enclosed .......................................................................................................................20

Figure 2-4: Planktonic bacteria (Pseudomonas fluorescens) suspended in liquid phase and the surface attached biofilm bacteria embedded in the self produced EPS ........................................................................................................22

Figure 2-5: Five stages of biofilm formation: A) initial attachment, B) irreversible attachment by EPS production, C) biofilm growth by cell division and EPS production, D) biofilm maturation, E) bacterial detachment and dispersion .....................................................................................................................................24

Figure 2-6: Illustration of the effect of biofilm on porous medium hydraulic properties. The extent of biofilm growth in pore spaces determines the extent of bioclogging and variations in the flow and transport properties of porous media ............................................................................................................30

Figure 2-7: Flow chamber used by Kildsgaard and Engesgaard (2002) (reproduced with permission – Appendix K) to study biofilm development in porous media under two-dimensional flow field. Tracer experiments were conducted to visualize flow path of nutrients within the porous medium. Evolution of tracer plume was also used to reveal flow field around bioclogged (i.e. biofilm saturated) regions ........................................36

Figure 2-8: A) Pseudomonas fluorescens biofilm formed inside a rectangular tube. Biofilm formation on the interior surface of pipes reduce the cross sectional area and reduce flow capacity, increasing friction and head...
loss. They also can contribute to corrosion in metal pipes. B) *Pseudomonas fluorescens* biofilm formed on a piece of steel plate. Formation of biofilm on surfaces in heat exchangers or cooling towers results in corrosion and heat transfer reduction ........................................42

Figure 2-9: By engineering biofilm structure and its growth pattern, permeable and impermeable biobarriers can be developed which can be utilized to remove pollutants and control the migration of contaminants in an aquifer .......................................................................................................44

Figure 2-10: Application of biofilm growth and bioclogging in microbial enhanced oil recovery (MEOR). Biofilm growth and development of impermeable biobarriers enhances the amount of recovered oil by bioclogging the preferential flow paths with high permeability. Therefore, the injected water can go through the low permeable regions and sweep the encapsulated trapped oils and push them through the production wells ........................................................................................................45

Figure 3-1: Controlling activities affecting biofilm development in a porous medium ........................................................................................................................................50

Figure 3-2: Water and biofilm phase relative permeabilities versus biofilm phase saturation ........................................................................................................................................58

Figure 3-3: Schematic of experimental apparatus used by Kildsgaard and Engesgaard (2002) - partially inoculated sandbox. The central 13 cm region (Inoculation Zone) was filled with sand that had been pre-inoculated with biofilm generating bacteria. A nutrient containing solution was injected into the sandbox at the bottom boundary (reproduced with permission – Appendix K) ............................................................................63

Figure 3-4: Schematic of experimental apparatus used by Thullner et al. (2004) - fully inoculated sandbox. The entire box was filled with sand that had been pre-inoculated with biofilm generating bacteria. A nutrient containing solution was injected into the sandbox at the bottom boundary (reproduced with permission - Appendix K) .............................65

Figure 3-5: Flow field around biofilm phase at different time intervals as marked by tracer injection (shown in dark black). Shown are (A) experimental results from Kildsgaard and Engesgaard (2002) (reproduced with permission) and; (B) results obtained from the new model. Tracer flow patterns reveal regions clogged due to biofilm growth and development. Area between the dotted lines shows the 13 cm inoculated region ..........70
Figure 3-6: Predictions of the biofilm phase saturation over time in Kildsgaard and Engesgaard’s (2002) experiment using the new model. The area between the two dotted lines represents the inoculation region where microbes were uniformly inoculated at the beginning of the simulation. Region A represents the downstream biofilm finger. Area between the dotted lines shows the 13 cm inoculated region ................................. 71

Figure 3-7: Prediction of relative permeability distribution of water phase after 30 days in Kildsgaard and Engesgaard’s (2002) experimental configuration. Region A represents the upstream section of the biofilm phase. Region B marks the downstream region of the biofilm phase whereas Region C embodies the downstream biofilm finger. Area between the dotted lines shows the 13 cm inoculated region .................. 73

Figure 3-8: Biofilm phase saturation at the end of the simulation and the biofilm phase effective permeability changes versus time at different points of the clogged zone in Kildsgaard and Engesgaard’s (2002) experimental configuration. Area between the dotted lines shows the 13 cm inoculated region .................................................. 75

Figure 3-9: Biofilm distribution near nutrient injection port: (A) Experimental results from Thullner and colleagues (2004) (reproduced with permission) where the biofilm saturated regions are visible by a light bank behind the sandbox (darker gray level corresponds to higher biofilm saturation), (B) Mathematical model predictions from Thullner and colleagues (2004) (reproduced with permission), and (C) predictions from the model developed here. Gray scale shows biofilm phase saturation ................................................................. 77

Figure 3-10: Tracer distribution near nutrient injection port: (A) experimental results from Thullner and colleagues (2004) (reproduced with permission) where dark plume represents color tracer travels in porous medium and bypasses the bioclogged regions (darker gray level corresponds to higher tracer concentration), (B) mathematical model predictions from Thullner and colleagues (2004) (reproduced with permission), and (C) predictions from model developed here. Gray scales represent relative tracer concentration (i.e. tracer concentration / initial tracer concentration at tracer injection port) ..................................................... 78

Figure 3-11: Model predictions of the biofilm development at the end of Kildsgaard and Engesgaard’s (2002) experiment (Day 30) for different relative permeability functions: (A) calibrated parameters (listed in Table 3-2), (B) higher water relative permeability values (L=1, E=1.5, T=4), (C) lower water relative permeability values (k_{rw}=0.7), (D) lower biofilm
phase relative permeability values ($k_{rbmax}=0.24$), and (E) higher biofilm phase relative permeability values ($k_{rbmax}=0.42$). Gray scale shows biofilm phase saturation. Area between the dotted lines shows the 13 cm inoculated region ................................................................. 80

Figure 4-1: Dark box used to provide completely dark environment to ensure minimal interference of bacterial bioluminescence by other light sources .................................................................................................................... 92

Figure 4-2: Experimental setup showing the custom designed flow chamber. The aluminum spacer was sandwiched between two transparent acrylic plates with gaskets serving to ensure a tight seal. The bottom acrylic plate was drilled with five holes in a row, located at 1, 3, 5, 7, and 9 cm from the inlet to monitor the local distribution of pressure along the chamber. The chamber was operated in a horizontal orientation. A Mariotte tube was used to house the biofilm growth medium used in the chamber. The inlet valves were used to ensure that, at any time, the medium only entered one porous medium channel, and not the other ........................................................................................................................................ 93

Figure 4-3: The porous medium flow chamber was divided into four zones as shown at the top of this figure. The flow inlet was located to the left of Zone 1, and the outlet was to the right of Zone 4. The chamber was inoculated on day zero at the interface between Zones 2 and 3 (marked by the circle). Bioluminescence images were obtained daily by introducing induction medium through the inlet, causing the cells in the biofilm to fluoresce. The bioluminescence images shown here represent the evolving biofilm growth pattern over the course of 8 days. Scale indicates increasing bioluminescence intensity from 0 to 6 .......... 97

Figure 4-4: Average number of bacterial cells in seven samples collected at the end of the experiment for each zone ................................................................................................................................. 105

Figure 4-5: Bioluminescence response of the biofilm in the porous medium over the course of 8 days by (A) induction from the left (inlet), and (B) induction from the right (outlet) side of the flow chamber. The chamber was initially inoculated at the interface between Zones 2 and 3 (marked by the circle). Scale indicates increasing bioluminescence intensity from 0 to 6 ................................................................. 108

Figure 4-6: (A) Overall bioluminescence image on Day 8 obtained by merging images captured following both inlet and outlet induction. Scale indicates increasing bioluminescence intensity from 0 to 6. (B) Binary
image obtained by applying the Otsu approach to determine a threshold value .................................................................109

Figure 4-7: Calculated average bioluminescence intensity for each zone during the course of 8 days experiment .................................................................110

Figure 4-8: Protein concentrations in seven samples collected at the end of the experiment for each zone .................................................................111

Figure 4-9: Photomicrographs of the biofilm samples obtained from upstream and downstream of the initially inoculated region ........................................112

Figure 4-10: Number of cells versus average bioluminescence intensity calculated on day 8 showing a power function relationship between cell numbers and average bioluminescence intensities at each zone .........................113

Figure 4-11: During the course of 8 days experiment, fluid flow rate through the porous medium, decreased gradually from 0.248 mL/min at the beginning to 0.110 mL/min by the end .........................................................116

Figure 4-12: Flow rates through the porous medium versus overall bioluminescence intensities calculated for the entire porous medium showing a linear correlation .................................................................116

Figure 4-13: Hydraulic head profiles measured along the porous media via pressure ports at the bottom of the flow chamber at 1, 3, 5, 7, and 9 cm from the inlet ........................................................................................................117

Figure 4-14: Hydraulic conductivity variations during the course of 8 days experiment combined with average bioluminescence intensities calculated for each zone showing possible correlations between average BI and porous medium hydraulic properties ..........................118

Figure 5-1: (A) Porous Package (PP) used to evaluate bacterial bioluminescence in porous media and liquid culture. Each PP was consisted of a cell strainer, filled with glass beads and placed in a Petri dish containing growth or induction medium. B) Bioluminescence image of a PP in a Petri dish. Simultaneous evaluation of planktonic cells bioluminescence in porous medium and planktonic cells in liquid culture in the same Petri dish allowed for a determination of the effect of porous medium on bacterial bioluminescence intensities ..................130

Figure 5-2: Packed tubes (PT), each consisting of 4 rectangular clear acrylic tubes (0.95 cm internal diameter) filled to a height of 2 cm with dried glass beads. PTs were used to grow biofilm under a constant pressure gradient and study hydraulic conductivity variations ..........................132
Figure 5-3: Biofilm volume was estimated from high resolution images of biofilm coated glass slides. Bacterial cells were allowed to attach and form biofilm for 12 days on the glass slide and subsequently, biofilm thickness and surface area of the covered glass slide was measured .... 134

Figure 5-4: Effect of oxygen saturation on bioluminescence intensity of planktonic cells of HK44 in induction medium which had been saturated with sterile air. Experiment conducted in batch mode, and the oxygen concentration and bioluminescence levels were simultaneously determined over time ............................................................... 137

Figure 5-5: Bioluminescence response of planktonic cultures of HK44 with different population densities in induction medium which had been fully saturated with pure oxygen. Higher oxygen saturation levels significantly increased the constant bioluminescence period by up to 360 minutes while keeping the maximum bioluminescence value unchanged .............................................................................. 139

Figure 5-6: Bioluminescence activity of planktonic cells in liquid culture and in porous medium indicating that the detected bioluminescence intensity of planktonic cells within the porous medium was lower than that exhibited by planktonic cells in liquid culture. Results indicated the applicability of lux gene dependent bioluminescence in evaluation of planktonic bacterial cell densities in liquid culture ......................... 140

Figure 5-7: Bioluminescence activity of biofilm cells. Based on the cell densities, lower bioluminescence activity was observed for the biofilm cells in porous medium .......................................................... 140

Figure 5-8: Regression analysis of data revealed a power relationship between cell density and detected bioluminescence intensity in all three sets of data for planktonic bacteria in liquid culture, planktonic bacteria in porous media, and biofilm bacteria in porous media ........................................... 141

Figure 5-9: Using biofilm dry density, biofilm saturation was evaluated gravimetrically in porous packages. The results revealed that a power function provides a good fit between calculated biofilm saturation and recorded bioluminescence intensity. Scale indicates increasing bioluminescence intensity from 0 to 16 ................................................. 143

Figure 5-10: Hydraulic conductivity of porous media versus bioluminescence intensity of each packed tube showing an exponential correlation ...... 144

Figure 5-11: Using the bioluminescence of bacterial cells, evolving biofilm saturation under flow conditions were assessed in packed tubes and plotted
against relative hydraulic conductivity. The results indicate that the published Clement model (dashed line) tends to overestimate hydraulic conductivities at similar biofilm saturation levels. However, an expression equivalent to the Clement model but with a higher exponent \( n = 6.3 \) provides a good fit..............................146

Figure 6-1: Stages in bacterial biofilm development: A) planktonic bacterial cells contact the solid surface; B) based on the contact angle and surface properties, some bacteria may reversibly attach to the solid surface; C) reversibly attached bacteria grow and proliferate through by cell duplication in the presence of sufficient nutrients; D) bacteria attach irreversibly by secretion of EPS and form a thin layer of biofilm; E) preliminary steps of biofilm maturation by initiating development of biofilm structure; and F) second maturation (fully mature biofilm) indicated by the complex biofilm structure and architecture ..............151

Figure 6-2: Presence of EPS on solid surfaces enhances bacterial attachment by: A) changing solid surface physicochemical properties (hydrophobic interactions) and B) increasing surface area for bacterial deposition (reducing available pore space and/or bridging the pore throats) ........153

Figure 6-3: Clear square acrylic tubes with inner diameter of 1.5 cm were used to conduct column experiments. Columns were filled up to 10 and 20 cm for fine (250 and 500 \( \mu\)m) and coarse (1 and 2 mm) glass beads ..........159

Figure 6-4: Tracer breakthrough curves in 4 packed beds with different sizes of glass beads (Table 6-1). Similar results were obtained in the other trial .................................................................167

Figure 6-5: Bacterial breakthrough curves combined with model prediction for bacterial transport in 4 packed beds with different sizes of glass beads (see Table 6-3 for details of each column). The general agreement between model predictions and experimental data indicates that the calculated sticking coefficients can be used to simulate bacterial migration in packed beds ..................................................................................169

Figure 6-6: Bioluminescence images captured throughout the breakthrough experiments to visualize microbial transport and fate in the packed beds (see Figure 6-3 for specifications of each packed bed). Bacterial bioluminescence detected after eluting with 3 PV sterile induction medium represents the attached bacteria in the packed beds. (B) Model predictions for bacterial transport in columns with different sizes of glass beads using the calculated attachment coefficients. Good agreement between model predictions and experimental data
indicates that the calculated sticking coefficients can be used to simulate bacterial transport behavior in packed beds

Figure 6-7: Bioluminescence images captured to visualize microbial transport and fate in porous media coated with biofilm. Initial bioluminescence in captured in each experiment indicates presence of biofilm (i.e. bacteria embedded in EPS) in packed bead. Higher final bacterial bioluminescence detected after eluting with 3 PV sterile induction medium indicates bacterial attachment in packed beds (see Figure 6-3 for specifications of each packed bed). (B) Model predictions for bacterial transport in biofilm coated packed beds using the calculated attachment coefficients. Good agreement between model predictions and experimental data indicates that the calculated sticking coefficients can be used to simulate bacterial migration in biofilm coated packed beds

Figure 6-8: Bacterial breakthrough curves combined with model prediction for bacterial transport in 4 packed beds with different sizes of glass beads coated with EPS. The general agreement between model predictions and experimental data indicates that the calculated sticking coefficients can be used to simulate bacterial migration in EPS coated packed beds

Figure 7-1: Biofilm can be engineered to form reactive permeable biobarriers to increase residence time and remediate subsurface contaminants. Also, impermeable biobarriers can be developed to prevent migration of contaminants to preserve groundwater resources

Figure 7-2: Application of bioclogging in microbial enhanced oil recovery. Biofilm growth and development of impermeable biobarriers enhances the amount of recovered oil by bioclogging the preferential flow paths with high permeability. As a result, the injected water will go through the less permeable regions and sweep the trapped oils and push them through the production wells

Figure 7-3: Custom designed flow chamber used to perform biofilm experiments under two-dimensional flow field in porous media. The flow chamber consisted of two main pieces; a base and a glass cover. The base was made of Plexiglas with a hole at the middle which had been removed to house porous media. The bottom of the Plexiglas base was drilled to fit ports for pressure evaluation all over the porous media. The ports are sealed with high-temperature silicon sealer to allow injection or sampling via syringe. Also, these ports could be used for inoculation and injection of solutes at any point within the porous media.
Fluorocarbon rubber O-ring (Viton®, Dupont Dow Elastomers) was used to seal the cover glass to the base by using C-clamps.

Figure 7-4: Schematic of the base of the flow chamber used in flow experiments to study biofilm evolution in porous media. The chamber base was made of 41 cm long, 23 cm wide, and 5 cm thick Plexiglas. A 25 cm by 14 cm by 1 cm hole was cut out of the base that was filled with glass beads to provide two-dimensional flow field to investigate biofilm behavior under flow conditions. The void space was outfitted with perforated plates, covered by steel mesh, to keep porous media in place. In addition, side holes were drilled at the side of the base to facilitate packing the flow chamber with glass beads. The chamber was also equipped with two inlet and outlet sockets to provide uniform fluid flow with minimal preferential flow paths within the porous media.

Figure 7-5: Dark box used in the experiments to capture bioluminescence images. Once the door was placed on the front of the box, it provided a completely dark environment to ensure minimal interference of bacterial bioluminescence by other light sources.

Figure 7-6: Flux changes during biofilm experiments in 3 different porous media (please see Table 7-1 for details of each porous medium). Values are means ± standard deviations (error bars).

Figure 7-7: Hydraulic pressure gradient across the flow chamber determined by averaging pressure readings at each distance from the inlet of the porous medium, facilitated by pressure ports at the bottom of the flow chamber base (please refer to Figure 7-4 for location of pressure ports at each level).

Figure 7-8: Comparison between overall relative permeability (compared to day 1) and relative permeability of upstream and downstream regions in 3 porous media A, B, and C (please check Table 7-1 for details of each medium). Values are means ± standard deviations (error bars).

Figure 7-9: Biofilm spatiotemporal development in 3 porous media (please see Figure 7-4 for details of the flow chamber). Arrows indicate flow direction in porous media.

Figure 7-10: Number of bacterial cells in porous media collected from upstream and downstream sampling regions at the end of flow experiments. Values are means ± standard deviations (error bars) of 12 samples obtained from each region.
Figure 7-11: Protein concentrations measured in porous media collected from upstream and downstream sampling regions at the end of flow experiments. Values are means ± standard deviations (error bars) of 12 samples obtained from each region ................................................. 206

Figure 7-12: Tracer movement in porous media A, B, and C during the course of 12 days experiment, indicating flow path of glucose and induction medium within porous media ............................................................... 210

Figure 7-13: Effect of biofilm on fluid flow in porous media with different grain sizes. In porous media with fine grain sizes (A and B) biofilm development can result in complete bioclogging, preventing penetration of fluid through biofilm regions. However, in porous medium with coarse beads (C), due to the moderately large pore sizes and high detachment rates, biofilm accumulation cannot continue to such extent that prevent flowing fluid from penetrating into biofilm regions .................................................................................................. 211

Figure 7-14: Biofilm development can have different effects on the permeability of a porous medium depending on the size of the pores. Shown here are illustrations of porous media with small (A) and large (B) pores. In porous media with fine particles, small pore sizes results in complete bioclogging, thereby impeding biofilm development at downstream regions. However, in porous media with moderately coarse particles, due to higher detachment rates, biofilm cannot completely plug pore spaces, resulting in biofilm dispersion at downstream regions ............ 213

Figure B-1: Growth curve of HK44 in growth medium used to calculate maximum growth rate coefficient ................................................................. 251

Figure E-1: Standard curve used to correlate HK44 population density to the light absorbance at 550 nm. Values are average absorbance with error bars based on five replicates for each cell concentration ............................... 255

Figure F-2: Regression analysis revealed strong linear correlation between mass of DNA and number of HK44 cells. The obtained correlation can be used to estimate HK44 population based on DNA measurements ....................... 259

Figure H-1: Standard curve used to determine protein concentration of biofilm samples ................................................................................................... 263

Figure I-1: Bioluminescence images obtained on Day 8 for two replicates of the one-way induction experiment that was described in the main text (Figure 4-3). It is evident that similar bioluminescence activities were observed in all replicates of the experiment (see Figure 4-3 of the main
text). In each case, bioluminescence was detected primarily in the upstream zones (Zones 1 and 2) towards the induction medium injection port. Significantly lower bioluminescence intensities were detected downstream of the initially inoculated region (in Zones 3 and 4). Scale indicates increasing bioluminescence intensity from 0 to 6.

Figure I-2: Shown here are the bioluminescence images obtained from one of the other one way induction trials. (A) Bioluminescence response of the cells indicating biofilm dispersion in porous medium over the course of 8 days. The induction medium was injected via the inlet located to the left of Zone 1, and discharged from the outlet located to the right of Zone 4. The chamber was inoculated at the interface between Zones 2 and 3. Scale indicates increasing bioluminescence intensity from 0 to 6. (B) Average number of bacterial cells in seven samples collected from each zone at the end of the experiment.

Figure I-3: (A) To show reproducibility of data in two-way induction experiments, the overall bioluminescence image obtained from a second trial is presented. The overall bioluminescence image was obtained by merging the images captured following both inlet and outlet induction on Day 8 of the experiment. Scale indicates increasing bioluminescence intensity from 0 to 6. (B) Number of cells versus average bioluminescence intensity calculated on Day 8. In Figure 4-10 from the main text, a power function relationship was derived using that set of data. The same power function relationship has been superimposed on this figure (dashed line). It is evident that the scaling exponent (equal to 3.11) obtained using the data in Figure 4-10 is also a good fit for the second set of data displayed here. (C) Flow rates through the porous medium versus overall bioluminescence intensities calculated for the entire porous medium. In Figure 4-12 from the main text, a linear relationship was derived using that set of data. The same linear correlation has been superimposed on this figure (dashed line). It is evident that the linear correlation obtained using the data in Figure 4-12 is also a good fit for the set of data displayed here.
# Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$a$</td>
<td>Specific surface area of spherical particles [1/L]</td>
</tr>
<tr>
<td>$A$</td>
<td>Cross sectional area to fluid flow [L$^2$]</td>
</tr>
<tr>
<td>$A_S$</td>
<td>Porosity dependent parameter [dimensionless]</td>
</tr>
<tr>
<td>$BI$</td>
<td>Bioluminescence intensity [arbitrary unit]</td>
</tr>
<tr>
<td>$C$</td>
<td>Concentration [m/L$^3$]</td>
</tr>
<tr>
<td>$\theta$</td>
<td>Cementation exponent</td>
</tr>
<tr>
<td>$d$</td>
<td>Diameter [L]</td>
</tr>
<tr>
<td>$D$</td>
<td>Hydrodynamic dispersion coefficient [L$^2$/t]</td>
</tr>
<tr>
<td>$D^M$</td>
<td>Molecular diffusion coefficient [L$^2$/t]</td>
</tr>
<tr>
<td>$D_{SE}$</td>
<td>Stokes-Einstein diffusion [dimensionless]</td>
</tr>
<tr>
<td>$g$</td>
<td>Gravitational acceleration [m/s$^2$]</td>
</tr>
<tr>
<td>$h$</td>
<td>Hydraulic head [L]</td>
</tr>
<tr>
<td>$L$</td>
<td>Length [L]</td>
</tr>
<tr>
<td>$\varphi$</td>
<td>Porosity of the porous medium [dimensionless]</td>
</tr>
<tr>
<td>$k$</td>
<td>permeability [L$^2$]</td>
</tr>
<tr>
<td>$K$</td>
<td>Hydraulic conductivity [L/t]</td>
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<tr>
<td>$H$</td>
<td>Hamaker Constant [ML$^2$/t$^2$]</td>
</tr>
<tr>
<td>$k$</td>
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<tr>
<td>$N_G$</td>
<td>Gravity number [dimensionless]</td>
</tr>
<tr>
<td>$N_{Pe}$</td>
<td>Peclet number [dimensionless]</td>
</tr>
<tr>
<td>$N_R$</td>
<td>Aspect ratio [dimensionless]</td>
</tr>
<tr>
<td>$N_{vdW}$</td>
<td>van der Waals number [dimensionless]</td>
</tr>
</tbody>
</table>
\( P \) Pressure \([\text{M/L}^2\text{t}^2]\)
\( q \) Volumetric flow rate \([\text{L}^3/\text{t}]\)
\( Q \) Volumetric injection/production rate \([\text{L}^3/\text{t}]\)
\( R \) Rate of reaction \([\text{M/L}^3\text{t}]\)
\( R_{Epb} \) Recovery of bacterial cells in effluent \([\text{dimensionless}]\)
\( S \) Saturation \([\text{dimensionless}]\)
\( t \) time \([\text{t}]\)
\( u \) Darcy velocity \([\text{L/t}]\)
\( v \) Fluid velocity in pore network \([\text{L/t}]\)
\( V \) Volume \([\text{L}^3]\)
\( Y_{bb/s} \) Yield coefficient of biomass production \([\text{dimensionless}]\)
\( Y_{EPS/Cbb} \) Yield coefficient of EPS formation \([\text{dimensionless}]\)
\( \nabla \) Gradient operator
\( \alpha_t \) Horizontal dispersivity \([\text{L}]\)
\( \alpha_l \) Vertical dispersivity \([\text{L}]\)
\( \epsilon_s \) Biofilm solid mass fraction \([\text{dimensionless}]\)
\( \epsilon_v \) Biofilm void fraction \([\text{dimensionless}]\)
\( \rho \) Density \([\text{m/L}^3]\)
\( \Theta \) Threshold value in bioluminescence images \([\text{arbitrary unit}]\)
\( \lambda \) Specific growth rate of bacteria \([1/\text{t}]\)
\( \lambda_{att} \) Attachment coefficient \([1/\text{t}]\)
\( \lambda_{det} \) Detachment coefficient \([1/\text{t}]\)
\( \lambda_q \) Bacterial quiescence coefficient \([1/\text{t}]\)
\( \eta_c \) Collision efficiency of a single collector \([\text{dimensionless}]\)
\( \eta_r \) Single-collector removal efficiency \([\text{dimensionless}]\)
\( \mu \) Viscosity \([\text{M/Lt}]\)
\( \varphi \) Porosity of the porous medium \([\text{dimensionless}]\)
\( \Omega \) Sticking efficiency \([\text{dimensionless}]\)
\( \tau \) Tortuosity \([\text{dimensionless}]\)
Subscripts

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>abs</td>
<td>Denotes absolute values</td>
</tr>
<tr>
<td>b</td>
<td>Denotes biofilm</td>
</tr>
<tr>
<td>bb</td>
<td>Denotes biofilm bacteria</td>
</tr>
<tr>
<td>db</td>
<td>Denotes dry biofilm</td>
</tr>
<tr>
<td>g</td>
<td>Denotes glass beads</td>
</tr>
<tr>
<td>i</td>
<td>Denotes phases</td>
</tr>
<tr>
<td>j</td>
<td>Denotes components</td>
</tr>
<tr>
<td>max</td>
<td>Denotes maximum value</td>
</tr>
<tr>
<td>n</td>
<td>Denotes nutrient</td>
</tr>
<tr>
<td>p</td>
<td>Denotes pore</td>
</tr>
<tr>
<td>pb</td>
<td>Denotes planktonic bacteria</td>
</tr>
<tr>
<td>r</td>
<td>Denotes relative values</td>
</tr>
<tr>
<td>s</td>
<td>Denotes substrate</td>
</tr>
<tr>
<td>w</td>
<td>Denotes water</td>
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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Trisphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BI_B</td>
<td>Background Bioluminescence Intensity</td>
</tr>
<tr>
<td>BI_O</td>
<td>Objective Bioluminescence Intensity</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BTC</td>
<td>Breakthrough Curve</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge Couple Device</td>
</tr>
<tr>
<td>CFT</td>
<td>Colloid Filtration Theory</td>
</tr>
<tr>
<td>CMG</td>
<td>Computer Modelling Group</td>
</tr>
<tr>
<td>CSLM</td>
<td>Confocal Scanning Laser Microscopy</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin, Landau, Verwey, and Overbeek theory</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double Stranded DNA</td>
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<tr>
<td>EPS</td>
<td>Extracellular Polymeric Substances</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LET</td>
<td>Lomeland, Ebeltoft, and Thomas relative permeability model</td>
</tr>
<tr>
<td>MEOR</td>
<td>Microbial Enhanced Oil Recovery</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MSM</td>
<td>Mineral Salts Medium</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PELB</td>
<td>Protein Extraction Lysis Buffer</td>
</tr>
<tr>
<td>PP</td>
<td>Porous Pack</td>
</tr>
<tr>
<td>PT</td>
<td>Porous Tube</td>
</tr>
<tr>
<td>PV</td>
<td>Pore Volume</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl Chloride</td>
</tr>
<tr>
<td>rcf</td>
<td>Relative Centrifugal Force</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>STANMOD</td>
<td>Studio of Analytical Models</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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</table>
Chapter 1

Introduction

1.1 Motivation and Goals

Mutual interaction between fluid flow and porous media hydraulic properties is a well described phenomenon (Bear, 1972; Scheidegger, 1974). Porous media hydraulic properties can change over time through different physical, chemical, and biological processes (Vandevivere and Baveye, 1992). For example, physical clogging is the main issue in filtration of suspended solids by porous structures. Precipitation of solutes on solid matrix of a porous medium is an example of a chemical process that can clog a porous medium and lower its permeability. Also, dissolution of solid matrix minerals when fluids with different chemical properties are injected can make porous media more permeable to fluid flow. When active microorganisms, including bacteria and fungi, enter a porous structure, biological processes, such as the attachment and growth of microorganisms can also alter the hydraulic properties of a porous medium by reducing the available pore space, and clogging the pore throats.

Under conditions conducive to cell growth, microorganisms that are planktonic (i.e. suspended in a liquid) can attach to the solid surface of a porous matrix and form biofilm by secretion of extracellular polymeric substances (EPS). Biofilms are structured communities of microorganisms encapsulated in a self-produced slime-like organic matrix of EPS. In porous structures, which inherently have high specific surface areas (i.e. high surface area to volume ratios), microbes can quickly attach to the surface of the porous matrix and start to form biofilm rather than remaining in a planktonic state (van
Loosdrecht et al., 1990; Bouwer et al., 2000). Under favorable growth conditions, biofilms can progressively accumulate within the void spaces (Taylor and Jaffe, 1990a; Cunningham et al., 1991; Baveye et al., 1998; Bozorg et al., 2012), making it increasingly difficult for fluids to flow through the porous medium.

The development of biofilm in porous media can impact key geohydrological characteristics of a pore network, such as effective porosity (the fraction of the pore space available to contribute to fluid flow) and permeability, thereby affecting fluid flow, mass transfer, and microbial activity in porous structures (Seki et al., 1998; Bielefeldt et al., 2002; Kildsgaard and Engesgaard, 2002; Thullner et al., 2002a; Arnon et al., 2005; Pavelic et al., 2007; Seifert and Engesgaard, 2007; Bozorg et al., 2012). Biofilm growth in porous media involves cell proliferation as well as secretion of EPS. As shown in Figure 1-1, an increase in the saturation of biofilm (i.e. an increase in the fraction of pore space occupied by bacterial cells and EPS) may plug pores and pore throats in porous

![Image](image_url)

**Figure 1-1:** Illustration of development of biofilm in porous media alters the hydraulic properties of porous structure. Increased amount of biofilm reduces the available pore space to fluid flow and may plug the pore throats, resulting in lower permeability (i.e. bioclogging).
media, thereby altering hydraulic properties (Figure 1-2). Such reduction in permeability due to microbial processes is referred to as biological clogging or bioclogging (Baveye et al., 1998).

Bioclogging occurs due to increasing biomass in the pore space as a result of metabolic activity, and therefore can be viewed as a reactive process. This process is controlled by the presence of nutrients carried by the fluid through the porous media (Gerlach and Cunningham, 2010), and thus, depends on geohydrological properties (e.g. permeability and porosity) of the porous medium itself. Since the dissolved components that affect cell survival and proliferation, such as nutrients and metabolic wastes, are carried by the fluids in porous media, variations in fluid flow patterns can influence the growth rate, structure, and spatiotemporal development of biofilm. Therefore, to understand bioclogging in porous media, mutual interactions between biological processes, fluid flow, and porous media hydraulic properties should be investigated.

![Figure 1-2: Bioclogging due to biofilm growth on solid particles in a porous medium (only 5 particles displayed) which can either reduce the available pore space to fluid flow or plugging the pore throats.](image)
Based on the negative impact that biofilms can have on porous media permeability, their formation is often considered to be detrimental and undesirable in industrial processes that rely upon the flow of fluids in porous media. For example, bioclogging has been found to adversely affect the performance of sand filters used to treat wastewater (Nicolella et al., 2000) and drinking water (Sharp et al., 2001; Urfer and Huck, 2001). However, engineering biofilms through the active management of their growth in porous media can provide significant opportunities to improve performance of processes that require biofilms to achieve a process goal, such as in situ bioremediation, reactive biobarriers, wastewater treatment, enhanced oil recovery, and carbon sequestration (Shaw et al., 1985; Harvey et al., 1989; Madsen, 1991; Lappin-Scott et al., 1998; Nicolella et al., 2000; Kim et al., 2006; Singh et al., 2006; Bishop, 2007; Mitchell et al., 2009). What is needed are methods that minimize the detrimental effects of biofilms in porous media while maximizing the potential benefits.

Development of robust methods to engineer biofilms in porous structures requires comprehensive knowledge of the processes that affect their spatiotemporal development under different flow conditions. However, due to an inadequate understanding of the interactions between geohydrological properties, biofilm growth kinetics, multiphase flow effects, spatial variations of cell nutrients, and the impact of medium heterogeneity, even the best models are not able to accurately predict observed biofilm behavior and geohydrological changes in porous media. Accordingly, field scale applications of biofilm based processes are still unpredictable (Bielefeldt et al., 2002a; Seifer and Engesgaard, 2007; Geesy and Mitchell, 2008; Gerlach and Cunningham, 2010). Different studies have investigated biofilm growth and several models have been developed to
interpret experimental results and predict biofilm behavior in porous structures (Baveye and Valocchi, 1989; Taylor and Jaffe, 1990c; Tan et al., 1994; Vandevivere et al., 1995; Clement et al., 1996; Noguera et al., 1999a,b; Dupin et al., 2001; Thullner et al., 2004; Brovelli et al., 2009; Bozorg et al., 2011). In such modeling attempts, biofilm has been treated as a growing solid phase capable of modifying intrinsic porosity and permeability of porous medium. Nonetheless, such models are limited due to inconsistencies in actual biofilm density and lack of reliable correlations between porosity and permeability.

To engineer biofilms in porous media, models of biofilm attachment, colonization, growth and spreading are required. In this thesis, the main focus was on the development of a new approach to simulate biofilm behavior and better understand its interactions with geohydrological properties of porous medium. Bioclogging effects were investigated theoretically by using numerical simulation and experimentally in the laboratory. As a novel approach, a new model of biofilm transport and growth in porous media was developed by treating biofilm as a highly viscous liquid that shares pore space with an immiscible low viscosity aqueous phase. In other words, biofilm development in porous media was modeled as a multiphase flow process and bioclogging effects were simulated using relative permeability curves. According to the relative permeability concept used in our model, the occupation of an increasing fraction of the pore space by the biofilm eventually drops the water relative permeability to zero, thereby resulting in bioclogging. Thus, instead of relying on explicit correlations between biofilm volume, porosity, and permeability, relative permeability curves were used to describe the two phases.

However, new parameters introduced in the mathematical model needed to be assessed experimentally. Therefore, to calibrate the new mathematical model and experimentally
evaluate its parameters, biofilm development was studied experimentally under one and two dimensional flow conditions. Additionally, evaluation of such parameters required concurrent evaluation of porous media hydraulic properties and biofilm characteristics. To accomplish this, a real-time visualization approach was needed to investigate the dynamic behaviors of biofilm in porous media. However, previous experimental approaches published in the literature for studying biofilms were not appropriate to properly monitor biofilm development in real time within porous media and determine its characteristics. Negative aspects of these previously published techniques, including the destructive nature of sampling, extensive time needed to provide samples, high energy exposure, lack of resolution, limited depth penetration, or selectivity have made them inappropriate for real-time visualization of biofilm development in porous media, and thus, not useful for investigating the effects of different parameters on evolving biofilm development. Therefore, we developed a novel real-time imaging method by using a biofilm forming bioluminescent bacterium, to simultaneously monitor biofilm development and evaluate its characteristics in porous medium. Furthermore, the captured bioluminescence intensities were linked to porous medium hydraulic properties as well as biofilm characteristics and used to assess such parameters nondestructively in real-time.

1.2 Problem Statement

To better understand biofilm behaviors and engineer its activities and growth pattern in porous media, comprehensive knowledge regarding bacterial attachment to solid surfaces, biofilm development, and mutual interactions between biofilm evolution and porous medium hydraulic properties are required. However, despite the published
theoretical, experimental, and numerical efforts, processes affecting biofilm development are not clearly understood and there are no complete answers to the following questions:

- How does biofilm development impact fluid flow through porous media?
- How does fluid flow affect biofilm development in porous media?
- How can evolving hydraulic properties be estimated and predicted during biofilm development in porous media?
- How can dynamic biofilm behaviors be simulated in porous structures?
- In a porous structure, how does the formation of biofilm affect microbial transport and further spreading of biofilm?
- Which parameters have an impact on biofilm spatial expansion in porous structures?

The overall objective of the research documented in this dissertation was to provide further understanding of biofilm development through a porous medium. Therefore, numerical and experimental studies were conducted to address the following main research objectives:

1. Develop a numerical model to simulate biofilm evolution in porous media.
2. Experimentally evaluate the effect of biofilm development on porous media permeability in one- and two-dimensional flow fields.
3. Develop an experimental setup to monitor biofilm evolution within porous media.
4. Nondestructively evaluate biofilm characteristics and porous media hydraulic properties in real-time.
5. Develop an experimentally-based expression for relative permeability based on biofilm saturation in porous media.
6. Evaluate the impact of biofilm on bacterial transport within a porous medium.
7. Conduct experiments to investigate influencing factors affecting biofilm spatial expansion in porous media.
1.3 Thesis Overview

Portions of the work in the present thesis have already been published, or are in the process of publication in peer-reviewed journals. Therefore, unavoidably, there will be some repetition between the Chapters, particularly in the Introduction section or in the sections dealing with the Materials and Methods. A short overview of the structure of this thesis is as following:

- Chapter 2 (*Literature Review*) presents a literature survey and describes the steps from initial biofilm formation to bioclogging. Biofilm applications in industry are discussed, and the beneficial and detrimental effects of biofilms are introduced. This literature review gives specific attention to biofilm development in porous media; more precisely influence of biofilms on porous media hydraulic properties.

- Chapter 3 (*A New Approach to Model the Spatiotemporal Development of Biofilm Phase in Porous Media*) introduces a new approach to model biofilm growth and development in porous medium by treating biofilm as a high viscosity liquid that interacts hydraulically with other fluid phases within the porous medium. Basic terms required for the description of such a model concept are described. A set of equations is derived using the mass conservation principle, and constitutive relations are determined to study the interactions between biofilm evolution and fluid flow in porous structures. However, as the proposed modelling approach was novel, actual data for newly introduced parameters were not available in literature, and had to be evaluated. The experimental approach is described in Chapters 4 – 7.
- **Chapter 4** *Real Time Monitoring of Biofilm Development under Flow Conditions in Porous Media* demonstrates an experimental approach that was used to simultaneously evaluate biofilm development and its corresponding effects on porous media hydraulic properties. Real-time imaging, in conjunction with a bioluminescent bacterial strain was used to monitor biofilm growth and spread within a translucent porous medium. Also, the developed imaging technique was used to correlate bioluminescence intensities to bacterial cell population and biofilm saturation (i.e. fraction of void space occupied by biofilm) as well as porous media hydraulic conductivity, thereby facilitating nondestructive evaluation of such parameters. Such an experimental approach was novel, and is essential to study dynamic biofilm behaviors in porous media.

- **Chapter 5**, *Application of Bacterial Bioluminescence in Porous Media to Evaluate Biofilm Characteristics and Hydrodynamic Properties*, shows the applicability of developed an imaging method, described in Chapter 4, to noninvasively evaluate biofilm characteristics and porous medium hydraulic properties in real-time. Mathematical correlations are provided to correlate biofilm and porous media characteristics to bioluminescence intensities. Using such correlations, the effect of biofilm saturation on fluid transport in porous media was investigated and relative hydraulic conductivity expression was developed.

- **Chapter 6**, *Effect of Extracellular Polymeric Substances on Bacterial Fate and Transport in Porous Media*, is concerned with the effect of biofilm on bacterial transport in porous media. Bacterial breakthrough experiments are conducted in columns packed with porous media of different hydraulic properties and the developed
imaging method discussed in Chapter 5 is utilized to study transport and deposition behavior of bacteria in the packed beds. Using colloidal filtration theory, bacterial attachment coefficients are calculated for clean and biofilm coated porous media and used to simulate fate and transport of bacteria in each column experiment.

- Chapter 7, *Effects of Porous Medium Hydraulic Properties on Biofilm Growth Patterns in Porous Media*, presents results of biofilm experiments conducted in a two-dimensional chamber filled with porous media of different hydraulic properties. The developed imaging technique, discussed in Chapters 4 and 5, is used to investigate how fluid flow, itself, impacts biofilm evolution in porous media. Biofilm spatiotemporal development was monitored and the different growth patterns observed in each porous medium are explained by diverse properties of the porous matrix. Also, tracer experiments were performed to illustrate variations in solute transport and flow path of nutrients within the porous media due to bioclogging.

- Chapter 8, *Conclusions and Recommendations*, summarizes the main results of the research documented in this thesis and also, identifies future research to advance the optimization of methods to engineer biofilms in porous structures.
Chapter 2

Literature Review

2.1 Overview

Formation of microbial biofilms in natural and engineered porous media can significantly influence the system hydrodynamics. Biofilm formation initiates by attachment of microorganisms to a substratum and evolves by their proliferation. As they proliferate, they also excrete extracellular polymeric substances (EPS) which encases them, resulting in a biofilm. In general, biofilms are more resistant to environmental stresses than free floating planktonic cells. Biofilm development in porous media impacts geohydrological properties such as porosity, hydraulic conductivity, and mass transfer of reactive and nonreactive solutes. Comprehensive knowledge of processes affecting biofilm characteristics, and engineering biofilm development in porous structures, can maximize potential benefits of porous media biofilms while minimizing detrimental effects of biofilms in processes that rely on fluid flow in porous media. Subsurface bioremediation, reactive biobarriers, microbial enhanced oil recovery (MEOR), and carbon sequestration are only some examples of beneficial porous media biofilm applications.

To understand biofilm process in porous media, a firm understanding of porous media, multiphase fluid flow in porous structures, geohydrological properties, and biofilm characteristics are required. Here, in the rest of this chapter, we introduce the basic terms and concepts of biofilm systems and a review of studies conducted in this area is presented.
2.2 Porous Media

The term porous medium (Figure 2-1) refers to a material consisting of a solid matrix with interconnected void space (pores) large enough to contain fluids, but small enough that interfacial forces control the location and shape of the interface between two fluids in the void space (Bear, 1967). Typical examples of porous media include soils, limestone, groundwater aquifers, petroleum reservoirs, living tissue, textiles, and wood. Porous media are usually characterized by porosity, pore size distribution, specific surface area, and permeability. Inherently, porous media have very high specific surface area, meaning that high surface area per unit volume is available for microbial attachment in porous structures. Therefore, within porous media, most of the microbial processes occur at the vicinity of solid surfaces where the sessile microorganisms are present as biofilm.

Figure 2-1: Porous media, a solid matrix with interconnected network of voids (pores) filled with a fluid
2.2.1 Porosity

A characteristic property of porous media is porosity ($\phi$) defined as:

$$\phi = \frac{Volume \ of \ void \ space}{Total \ volume \ of \ porous \ medium} \tag{2.1}$$

Therefore, porosity is the ratio of pore volume to the total volume of a porous medium. For a clean porous medium filled with a fluid, porosity can be easily defined, measured, or calculated. However, calculating the porosity in the presence of biofilm is not straightforward, as biofilm is composed of more than 90% water, making it difficult to properly determine porosity. Also, evaluation of biofilm volume, which depends on the definition of the biofilm thickness, is another source of uncertainty. In general, height of the tallest cluster in a biofilm layer from the solid surface is used as the biofilm thickness (Vandevivere et al., 1995; Seki and Miyazaki, 2001), which can significantly overestimate volume of biofilm in porous media.

2.2.2 Phase Saturation

Saturation of a phase in a porous medium is the volume fraction of the pore space that is occupied by that specific phase. For example in an oil reservoir, oil saturation indicates the portion of pore space occupied by the oil phase.

2.2.3 Absolute Permeability

Absolute (i.e. intrinsic) permeability, which is related to the geometry of the pore network and the porosity of the medium, indicates the ease with which fluid can flow through the porous medium (Collins, 1961). Permeability, expressed in dimensions of $L^2$ (i.e. length squared) is the most frequently used parameter in describing the impact of
biofilm formation on porous media properties. A commonly used unit of permeability is the *Darcy*, after Henry Darcy, where 1 Darcy = $9.87 \times 10^{-9}$ cm$^2$ ≈ 1μm$^2$. In some published research papers however, hydraulic conductivity (which is easier to be measured experimentally) has been used instead of permeability. Conversion between these two parameters is simple and uses fluid properties as follows:

$$k = K \frac{\mu}{\rho g}$$  \hspace{1cm} (2.2)

where, $k$ is permeability, $K$ is hydraulic conductivity, $\mu$ is fluid viscosity, $\rho$ is fluid density, and $g$ is gravitational acceleration.

### 2.2.4 Darcy Equation

In 1856, Henry Darcy described a relationship between the fluid flow and pressure gradient of one-phase flow through porous structures, later to be known as the Darcy equation. The Darcy equation is a phenomenological macroscopic description of fluid flow within porous structures which is widely used in the study of single-phase flow through porous media. However, the Darcy equation has also been generalized to model multi-phase flow. The differential form of Darcy’s equation for single-phase flow in pore networks is given as (Collins, 1961):

$$u = \frac{q}{A} = -\frac{k}{\mu} (\nabla P + \mu g)$$  \hspace{1cm} (2.3)

where $u$ indicates Darcy velocity, $q$ is the volumetric flow-rate through porous media, $A$ is cross sectional area, $k$ is absolute permeability, $\nabla P$ is pressure gradient across the porous media, $\rho$ is density of fluid, $g$ is gravitational acceleration, and $\mu$ is fluid viscosity,
indicating fluid resistance to flow. Note that the actual fluid velocity \((v)\) in pore network in different from Darcy velocity and can be calculated as:

\[
v = \frac{u}{\varphi}
\]  

(2.4)

As the porosity is always less than unity, the actual velocity is always higher than Darcy velocity in porous media.

The following generalized form of Darcy equation has been proposed for multi-phase flow in porous media:

\[
\mathbf{u}_i = -\frac{k_i^e}{\mu_i} (\nabla P + \rho_i g)
\]  

(2.5)

where \(k_i^e\) represents effective permeability of phase \(i\) within the pore network. Contrary to the absolute permeability, when more than one fluid phase flows through the porous medium, the effective permeability depends not only on the pore geometry, but also on the fraction of void space has been occupied by the other phases. It means that, in general, when multiple fluid phases share pore space in a porous medium, there is an increased resistance to the flow of any one phase due to the reduced available pore space for its flow.

Therefore, consider multiphase flowing, the effective permeability \((k_i^e)\) of any phases depends on saturation of its phase in pore spaces, meaning that higher saturation of any phase increase its effective permeability in porous media (i.e. reduce restrictions related to the presence of other phases). It is common to normalize the effective permeability by the absolute permeability:
\[ k_i^r = \frac{k_i^e}{k_i} \quad \left(0 < k_i^r \leq 1\right) \]  

(2.6)

where the dimensionless quantity \( k_i^r \) is phase \( i \) relative permeability. Thus, relative permeability can be used to modify the absolute permeability of porous media to add restrictions to flow of a specific fluid in multiphase fluid flow in a pore network.

### 2.3 Bacteria

Bacteria are a diverse group of more than 2000 species. Different bacteria possess a vast variety of metabolic enzymes, which enables them to survive under diverse environmental conditions and drive nutrients from different sources of food and energy. Therefore, bacteria can be found in almost every ecosystem (the air, soil, water). Bacteria are the simplest single-celled microorganisms belonging to the group of living organisms called prokaryotes. Morphologically, most bacteria are either rod-shaped (i.e. bacillus), or spherical (i.e. cocci) with an average of 0.5 \( \mu \text{m} \) to 5 \( \mu \text{m} \) in length (Tchobanoglous et al., 2003). As prokaryotes, bacteria lack a defined cell nucleus or even any other membrane-bounded cytoplasmic organelles, such as mitochondria or chloroplasts, and possess a single, circular DNA molecule (i.e. chromosome) including all the necessary genes. However, some species (e.g. genetically engineered bacteria) contain a smaller piece of DNA, called a plasmid, which usually encodes non-essential functions and provide the bacteria with specific capabilities (Figure 2-2). Bacteria grow through a simple asexual process of binary fission, whereby a single cell (i.e. mother cell) grows to a fixed size and doubles the quantity of intracellular components prior to splitting into two daughter cells.
Under optimal growth conditions, bacterial population can typically duplicate every 20 to 30 minutes. There are four main structures shared by all bacterial species (Figure 2-2): plasma membrane, cytoplasm, ribosomes, and genetic material (DNA and RNA). In addition, many bacteria generate extracellular filamentous appendages, such as flagella and pili (or fimbriae) which are involved in the process of attachment to a solid surface (Harbron and Kent, 1988). Characteristics of typical bacterial cell structures are summarized in Table 2-1.
Table 2-1: Summary of typical bacterial cell structures

<table>
<thead>
<tr>
<th>Structure</th>
<th>Function</th>
<th>Main Chemical Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsule</td>
<td>- Protection</td>
<td>Polysaccharide</td>
</tr>
<tr>
<td></td>
<td>- Aiding surface attachment and biofilm formation</td>
<td></td>
</tr>
<tr>
<td>Cell Wall</td>
<td>- Prevent osmotic lysis of cell</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td></td>
<td>- Confers rigidity and shape</td>
<td></td>
</tr>
<tr>
<td>Plasma Membrane</td>
<td>- Permeability barrier to control what gets in and out of the cell</td>
<td>Phospholipid and protein</td>
</tr>
<tr>
<td></td>
<td>- Transport of ions, nutrients, and wastes via protein channels</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Holding all cell organelles</td>
<td>Water, salt, and protein</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>Protein synthesis</td>
<td>RNA and protein</td>
</tr>
<tr>
<td>Inclusions</td>
<td>Stores nutrients</td>
<td>Lipid, protein, carbohydrate, or inorganic material</td>
</tr>
<tr>
<td>Chromosome</td>
<td>Genetic material of the cell</td>
<td>DNA</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Extrachromosomal genetic material</td>
<td>DNA</td>
</tr>
<tr>
<td>Flagella</td>
<td>Swimming Movement</td>
<td>Protein</td>
</tr>
<tr>
<td>Pili</td>
<td>Attachment and protection</td>
<td>Protein</td>
</tr>
</tbody>
</table>

Flagella, if present, are responsible for bacterial motility. These are minuscule (less than 0.02 μm in diameter and a length of up to 10 μm) threads of the protein flagellin which has a helical structure that protrudes from the cell wall. It has been reported that the flagellum, by itself, can form an adhesive bond with a surface (Harbron and Kent, 1988). In biofilm formation, the primary role of flagella would be in bacterial movement and in initial cell-to-surface interactions (Sauer and Camper, 2001). Also, flagella-mediated motility is supposed to overcome repulsive forces at the surface of the substratum and thus, facilitates formation of a monolayer of attached cells on the solid surfaces (Daniels...
et al., 2004). Similar to flagella, pili are microscopic filamentous appendages of protein (4 to 35 nm wide and up to several micrometers long) found on many Gram-negative bacteria. Although not involved in motility, pili and pilus-associated structures can make cells more adhesive, probably by overcoming the initial electrostatic repulsion barrier that exists between cells and the substratum (Donlan, 2002). Bacteria with pili can strongly hold to each other, and adhere to inorganic particles (Sauer and Camper, 2001).

2.4 Biofilms – advanced microbial communities

Although bacteria can live as planktonic cells (i.e. suspended or free floating cells in a bulk fluid), in nature, they tend to attach to solid surfaces, multiply and embed themselves in a slime-like matrix, resulting in biofilm (Figure 2-3). It is believed that most microorganisms are organized in well structured biofilms, which provides an opportunity for them to survive in extreme environments such as hydrothermal vents, disinfection pipelines, and nuclear power plants (Costerton et al., 1995).

Biofilm was first described by Anthony van Leeuwenhoek in 1683 as an organic layer of living microorganisms formed on human teeth (see http://www.ucmp.berkeley.edu/history/leeuwenhoek.html [accessed March 2013] or Dixon, 2009). However, as surface attached microorganisms are very difficult to study, research conducted in the 20th century, were primarily focused on the study of planktonic (i.e. free floating suspended) microorganisms rather than sessile biofilms. In 1943, Zobell observed microbial cells attached in multiple layers to bottle walls and indicated that microbial cells in such layers are more resistant to cleaning. Later, Atkinson and his
Figure 2-3: Bacterial biofilm formed on a piece of glass slide. Bacterial cells permanently attach to the solid surface by secretion of EPS in which they are enclosed.

colleagues (1967) introduced the term "biological film" to represent the sticky gelatinous layer of cells formed on the surface of a bioreactor vessel. Also, in 1971, Topiwala and Hamer referred to the viscid layer of bacterial cells attached to glassware using their extracellular polymeric substances as "wall growth". Nevertheless, the importance of sessile microorganisms in natural and engineered systems was not really considered until the 1970s when Charackhilis (1973) clearly described the abundance of biofilms in various
environments. He provided an extensive survey on the fundamental and practical implications of “microbial slimes”. Finally, a consensus was reached by the leaders in the area in 1984 to define a biofilm as a community of microorganisms, predominantly bacteria, surrounded by their self-produced viscous matrix of extracellular polymeric substances (Marshall, 1984). It took until 1990 for the first book on biofilms to publish where two of the pioneers in biofilm research, Marshall and Characklis, published Biofilms, a book that is still valuable to novices in biofilm engineer and microbiology (Characklis and Marshall, 1990).

A biofilm can be defined as a structured community of microbial cells embedded in a self produced three-dimensional organic matrix of cellular products, like extracellular polymeric substances (EPS), attached to a solid surface (Figure 2-4). Biofilms are the predominant form of microbial life in different environments. It has been argued that more than 99% of bacteria found in moist environments exist as biofilms (Costerton, 1995), whether in the form of small separated attached colonies in nutrient poor environments, or as a continuous layer under more favorable growth conditions. Biofilms can be found on different surfaces and in various industrially, environmentally, and medically relevant systems, especially in media with high surface area to volume ratios, such as porous structures (VanLoosdrecht et al., 1990; Bouwer et al., 2000). They form in completely saturated aqueous systems as well as unsaturated moist environments such as pipelines, soils, medical implants, blood vessels, biomaterials, tissues, biofilters, cooling towers, ship hulls, oil reservoirs, and riversides.
Bacteria form biofilm to increase their resistance and protect themselves from various environmental stresses. To prevent mechanical washout under flow conditions, bacteria form biofilm on solid surfaces by secretion of EPS to irreversibly attach to the solid support. Also, embedding themselves in EPS provides an opportunity for them to improve their resistance to toxic materials, protect themselves from predators, and also aids in trapping nutrients in systems where nutrients are in low concentrations (Characklis and Cooksay, 1983). In general, formation of biofilms creates an
encapsulated and structured community of cells in which environmental stresses are greatly reduced (Anwar et al., 1992). Therefore, biofilm bacteria predominate numerically and metabolically in almost all moist environments with sufficient nutrient levels and accordingly, can influence performance of different environmental, industrial, and medical processes.

2.4.1 Biofilm Formation

The stages that lead to development of mature biofilm have been well investigated (Costerton and Lappin-Scott, 1995). In biofilm, bacteria exhibit more complicated behaviors than suspended planktonic cells, and once established, the biofilm can influence the surrounding ecosystem. The formation of a bacterial biofilm initiates by attachment of planktonic bacteria to a solid surface in a moist environment (Figure 2-5). Planktonic bacterial cells floating in a fluid phase can be transported toward a solid surface by different transport processes such as diffusive and convective forces, and/or active movement. Some of the cells in contact with the substratum adsorb to it. In most cases, prior to bacterial attachment, an organic monolayer need to form on the solid surface by adsorption of dissolved organic molecules. The adsorbed organic compounds condition the solid surface by changing its physicochemical properties and increase the probability of a successful bacterial attachment. Also, this conditioning layer, which is usually composed of polysaccharides and glycoproteins, can be a concentrated nutrient source for the adsorbed bacteria (Lewandowski and Cunnigham, 1998).
Bacterial adsorption can be reversible or irreversible. The first cells attach reversibly to the surface, initially by van der Waals forces. However, if association between bacterial colonies and the solid surface persist long enough, they can anchor themselves more securely by producing EPS. This means that, adsorbed cells secrete EPS to ensure their attachment to the substratum and to one another (Lewandowski and Cunningham, 1998). Once colonized, the biofilm grows through a combination of cell proliferation and EPS secretion in the presence of sufficient nutrients and favorable growth conditions. At this stage in which the biofilm is established, further development by cell division and EPS production may only change the shape and size of the mature biofilm. Finally, detachment and dispersion of bacteria from the mature biofilm can occur due to erosion (i.e. detachment of single cells) and sloughing (i.e. detachment of biofilm pieces). Bacterial detachment/dispersion occurs due to interfacial shear stresses exerted by the bulk fluid on the biofilm surface and can lead to continuous cell detachment from the

Figure 2-5: Five stages of biofilm formation: A) initial attachment, B) irreversible attachment by EPS production, C) biofilm growth by cell division and EPS production, D) biofilm maturation, E) bacterial detachment and dispersion
biofilm. Enzymes that degrade biofilm EPS, such as dispersin B and deoxyribonuclease, can reduce biofilm matrix integrity and increase bacterial detachment and biofilm dispersal.

2.4.2 Biofilm Composition

Biofilm can be divided into two main compartments; microbial cells, and biofilm matrix which is made of EPS. Based on the growth conditions and environmental factors, biofilm matrix composition and architecture can vary significantly from one biofilm to another. For example, biofilm growth in nutrient-rich environments and in favorable growth conditions results in spontaneous and poorly structured matrix whereas harsh conditions lead to highly structured communities (Characklis and Marshall, 1990). However, some aspects of their global composition are similar in biofilm phenotype. As shown in Table 2-2, a biofilm matrix is mostly composed of water (97% of total mass). The water can be bounded within the microbial cells or can present as an extracellular solvent whose physical properties such as viscosity should be determined based on its dissolved components (Sutherland, 2001).
Table 2-2: General composition of biofilms (Sutherland, 2001)

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbial cells</td>
<td>2-5%</td>
</tr>
<tr>
<td>Water</td>
<td>As high as 97%</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>2-5%</td>
</tr>
<tr>
<td>Proteins</td>
<td>2-4%</td>
</tr>
<tr>
<td>DNA – RNA</td>
<td>&lt; 2%</td>
</tr>
<tr>
<td>Ions</td>
<td>Negligible</td>
</tr>
<tr>
<td>Inorganics</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

2.4.3 Biofilm Matrix

Biofilms are composed of a viscid matrix in which microorganisms colonize (Sutherland, 2001). Biofilm matrix itself is composed of extracellular polymeric substances of biological origin that participate in the formation of cell aggregates or layer of biofilm. According to Table 2-2, bacterial biofilms EPS is, in general, composed of water, polysaccharides, proteins, nucleic acids, lipids, and phospholipids (Sutherland, 2001). Formation of EPS matrix, as well as its composition, can result from different processes including active secretion by the cells, shedding of microbial surface materials, and adsorption of environmental substances. In addition, death and lysis of microbial cells contribute to release of intracellular substances into the medium, which can be entrapped in biofilm matrix (Wingender et al., 1999).

In biofilms, EPS is responsible for structural coherence and adhesion to substratum (Kreft and Wimpenny, 2001; Sutherland, 2001; Allison, 2003). The EPS, as the main structural
elements of biofilm matrix determine the mechanical stability of biofilms. Non-covalent interactions, either directly between polysaccharides or indirectly via multivalent cation bridges, mediate structural integrity of biofilm matrix (Allison, 2003; Flemming and Wingender, 2010). Also, lectin proteins can also contribute, in part, in formation of three-dimensional structure of biofilm matrix by cross-linking polysaccharides through multivalent cation bridges. In some cases, where proteins predominate, a high content of negatively charged amino acids in protein molecules are supposed to be more involved than sugars in electrostatic bonds with multivalent cations (Dignac et al., 1998). However, main function of proteins in the established microbial community is providing sessile microbial cells with low molecular weight substrates, which can be readily be consumed and metabolized, by enzymatic digestion of macromolecules and particulate substances (Allison, 2003). Enzymes in biofilm matrix may also be involved in degradation of polysaccharides resulting in release and spread of biofilm bacteria to surrounding environment (Figure 2-5).

Functions of the other EPS components are not well recognized and remain to be established (Wingender et al., 1999). It is expected that lipids and nucleic acids affect biofilm rheological properties. Also, it is reported that DNA release is required during the initial stages of biofilm development (Whitchurch et al., 2002; Allesen-Holm et al., 2006). Table 2-3 summarizes the main functions of EPS in bacterial biofilms.
### Table 2-3: EPS related functions in bacterial biofilms (adapted from Wingender et al., 1999)

<table>
<thead>
<tr>
<th>EPS Function</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Surface adhesion</strong></td>
<td>- Initial step in biofilm formation</td>
</tr>
<tr>
<td></td>
<td>- Accumulation of bacteria on surfaces</td>
</tr>
<tr>
<td></td>
<td>- Protect from mechanical stresses</td>
</tr>
<tr>
<td></td>
<td>- Prevent bacterial washout</td>
</tr>
<tr>
<td><strong>Structure coherence</strong></td>
<td>- Determine the shape of biofilm matrix</td>
</tr>
<tr>
<td></td>
<td>- Mediation of biofilm matrix mechanical stability</td>
</tr>
<tr>
<td></td>
<td>- Formation of cell aggregates and biofilm</td>
</tr>
<tr>
<td></td>
<td>- Immobilization of bacterial</td>
</tr>
<tr>
<td></td>
<td>- Formation of microbial communities</td>
</tr>
<tr>
<td></td>
<td>- Development of high cell densities</td>
</tr>
<tr>
<td></td>
<td>- Providing communication network between cells</td>
</tr>
<tr>
<td></td>
<td>- Biocorrosion in process equipments</td>
</tr>
<tr>
<td></td>
<td>- Bioclogging of porous media</td>
</tr>
<tr>
<td></td>
<td>- Biofouling in industrial processes</td>
</tr>
<tr>
<td><strong>Cell-cell recognition</strong></td>
<td>- Symbiotic relationships with other organisms</td>
</tr>
<tr>
<td></td>
<td>- Pathogenicity</td>
</tr>
<tr>
<td><strong>Enzymatic activities</strong></td>
<td>- Digestion of macromolecules for nutrient acquisition</td>
</tr>
<tr>
<td></td>
<td>- EPS degradation and bacterial dispersion in environment</td>
</tr>
<tr>
<td><strong>Protective barrier</strong></td>
<td>- Protect bacteria from predation</td>
</tr>
<tr>
<td></td>
<td>- Resistance to biocides</td>
</tr>
<tr>
<td><strong>Sorption of organic compounds</strong></td>
<td>- Accumulation of substrates and scavenging of nutrients</td>
</tr>
<tr>
<td><strong>Sorption of inorganic ions</strong></td>
<td>- Accumulation of toxic ions</td>
</tr>
<tr>
<td></td>
<td>- Promotion of mineral formation</td>
</tr>
</tbody>
</table>
2.5 Bioclogging

In porous media, bacterial growth and biofilm accumulation can lead to clogging of pore spaces and thereby, resulting in significant changes in fluid flow and hydraulic properties of porous media (Tayloe et al., 1990a,c; Cunningham et al., 1990; Vandevivere and Baveye, 1992; Baveye et al., 1998; Seifer and Engesgaard, 2007). Therefore, to design and conduct successful biological processes in porous structures, careful attention should be given to possibility of accumulation of biological substances and the consequent permeability reduction due to bioclogging of pore spaces. Bioclogging can result in reduced porosity and permeability by modifying the geometry and size of available pore spaces (Baveye et al., 1998; Brovelli et al., 2009). A comprehensive summary of different types of bioclogging processes in porous media has been provided by Baveye and colleagues (1998). The bioclogging process is believed to occur during microbial growth and biofilm accumulation (Figure 2-6). The rate of biofilm development and increase in biofilm saturation in pore spaces determines the rate and extent of bioclogging, and accordingly, the rate and extent of variation of hydraulic properties of porous media (Vandevivere and Baveye 1992; Baveye et al., 1998).

Permeability reductions up to three orders of magnitude have been reported in experimental studies (VanGulck and Rowe, 2004). Bioclogging, similar to biofilm accumulation, is usually not homogeneous all over a porous medium and is spatially and temporally varied with higher production of biomass (microbial cells or EPS) at the regions with higher nutrient availability such as the area adjacent to the nutrient injection wells (VanGulck and Rowe, 2004). Also, bioclogging seems to be more pronounced in
porous media with fine particles and small pore sizes rather than coarse-textured ones (Vandevivere et al., 1995). Once bioclogged, due to the large amount of EPS which cannot be degraded readily, decreased permeability can often be maintained even during starvation or disinfection processes (Cunningham et al., 1991; Kim and Fogler, 2000; Kim et al., 2006). These observations have been confirmed by Kim and Fogler who observed no EPS degradation in batch experiments for a period of about 2 years (Kim and Fogler, 2000).

Table 2-4 summarizes permeability variations in biofilm experiments to compare bioclogging results of different studies in which microbial growth has affected the porous media permeability.
Table 2-4: Bioclogging results in different experiments indicating the influence of biofilm growth on porous media permeability reduction. Results indicate maximum permeability reductions achieved in bioclogging experiments.

<table>
<thead>
<tr>
<th>Initial Permeability (cm$^2$)</th>
<th>Log Reduction</th>
<th>Porous Media</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.42 x 10^{-8}</td>
<td>0.1</td>
<td>Glass beads</td>
<td>MacLeod et al., 1988</td>
</tr>
<tr>
<td>3.49 x 10^{-6}</td>
<td>0.2</td>
<td>Berea sandstone</td>
<td>Kalish et al., 1964</td>
</tr>
<tr>
<td>1.75 x 10^{-4}</td>
<td>0.2</td>
<td>Sand</td>
<td>Komlos et al., 2004</td>
</tr>
<tr>
<td>3.49 x 10^{-7}</td>
<td>0.3</td>
<td>Berea sandstone</td>
<td>Kalish et al., 1964</td>
</tr>
<tr>
<td>6.17 x 10^{-8}</td>
<td>0.4</td>
<td>Glass beads</td>
<td>Bozorg et al., 2011</td>
</tr>
<tr>
<td>3.49 x 10^{-6}</td>
<td>0.5</td>
<td>Berea sandstone</td>
<td>Kalish et al., 1964</td>
</tr>
<tr>
<td>6.42 x 10^{-8}</td>
<td>0.5</td>
<td>Glass beads</td>
<td>MacLeod et al., 1988</td>
</tr>
<tr>
<td>2.33 x 10^{-7}</td>
<td>0.6</td>
<td>Berea sandstone</td>
<td>Kalish et al., 1964</td>
</tr>
<tr>
<td>1.75 x 10^{-4}</td>
<td>0.7</td>
<td>Sand</td>
<td>Komlos et al., 2004</td>
</tr>
<tr>
<td>3.49 x 10^{-6}</td>
<td>0.9</td>
<td>Berea sandstone</td>
<td>Raiders et al., 1986</td>
</tr>
<tr>
<td>2.33 x 10^{-7}</td>
<td>1.0</td>
<td>Berea sandstone</td>
<td>Kalish et al., 1964</td>
</tr>
<tr>
<td>1.14 x 10^{-5}</td>
<td>1.0</td>
<td>Sand</td>
<td>Kildsgaard and Engesgaard, 2001</td>
</tr>
<tr>
<td>4.65 x 10^{-5}</td>
<td>1.1</td>
<td>Sand</td>
<td>Cusack et al., 1992</td>
</tr>
<tr>
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2.6 Approaches and Techniques for Studying Porous Media Biofilms

Different experimental systems have been used to characterize flow within and around biofilm, which has accumulated within a porous medium (deBeer et al., 1994; Sharp et al., 1999; Kildsgaard and Engesgaard, 2002; Seymour et al., 2004b; Seki et al., 2006; Seifert and Engesgaard, 2007) and a variety of reactor designs can also be imagined for investigation of biofilm processes in porous media. However, actual design of laboratory or industrial scale reactors is dependent upon the ultimate goal of each study or application. In porous media, the activity of biofilm bacteria is governed by availability of nutrients. Therefore, biofilm evolution in porous media is governed by different mass transfer processes, such as convection of solvents and solutes, possible mass transfer from the gaseous to the liquid phase (e.g., for oxygen as an electron acceptor), dispersion of solutes through bioclogged sections with low permeability, and simultaneous reaction, diffusion, and sorption of solutes within the biofilm and porous medium (Sarkar et al., 1994; Hunt et al., 2004; Sharp et al., 2005; Kim et al., 2000). It should be acknowledged that the activity of microorganisms in porous media will ultimately determine the success of biofilm technologies, and thus, determination of microbial activities should be utilized as much as possible. Therefore, experimental systems are required in which hydrodynamics and biological activity of biofilm bacteria can be measured simultaneously in real-time.

However, evaluation of biofilm activity spatially and temporally is a big challenge even in the absence of porous media. General approaches for determining biofilm activities include traditional microbiological culturing techniques (e.g., most probable number techniques or plate counts), consumption rate of nutrients, enzyme assays to evaluate
specific activities, and molecular techniques such as mRNA (messenger ribonucleic acid) measurements, PCR (polymerase chain reaction) for gene-specific quantification, and utilization of reporter gene in biofilm bacteria (Dorn et al., 2004; Geesey and Mitchell, 2008; Lenz et al., 2008).

2.6.1. Biofilm Imaging in Porous Media

Imaging biofilm within porous media is an ongoing challenge in biofilm studies. The ability to monitor biofilm in porous media would considerably facilitate validation of the theoretical models that have been developed to simulate biofilm behavior in porous structures. Moreover, in order to develop effective porous media biofilm applications and evaluate the effect of different parameters on biofilm behavior, biofilm spatiotemporal growth and spread as well as activity within the porous media should be monitored in real-time. Nevertheless, due to the opaque nature and irregular shape of porous structures, direct visualization of biofilm processes in porous media is a big challenge in experimental investigations. Therefore, development of new and improved experimental techniques for visualization of biofilm in porous media is an ongoing research topic in the field of biofilm engineering.

Destructive (i.e. invasive) and nondestructive measurements have been used to evaluate presence, spread, and structure of biofilms in porous media. Such techniques include visual imaging using high-resolution photography and microscopy, scanning or transmission electron microscopy (SEM or TEM), as well as more recently developed methods such as X-ray tomography, nuclear magnetic resonance (NMR) spectroscopy, or ultrasound-based imaging techniques. Low-energy and noninvasive techniques such as ultrasound imaging, optical microscopy, high resolution photography, or NMR imaging
have been used in biofilm research and although they suffer from a lack of resolution, selectivity, and depth penetration, utilization of such techniques is favorable due to their negligible impacts on living organisms.

In most experimental studies, biofilm development is inferred indirectly by monitoring its effects on porous media hydraulic properties, using standard soil methods (Vandevivere and Baveye, 1992; Cunningham et al., 1991, 1997), dye tracer experiments (Thullner et al., 2002; Kildsgaard and Engesgaard, 2002; Seki et al., 2006), and breakthrough studies (Wollum and Cassel, 1978; Harvey et al., 1989; Sharp et al., 1999). The nondestructive real-time method to evaluate biofilm phase accumulation in saturated porous medium was developed by Sharp et al. (1999) and Kildsgaard and Engesgaard (2002), by considering effects of biofilm phase development on porous media hydraulic properties (i.e. bioclogging).

Sharp et al. (1999) used dye tracer experiments and advanced image analysis to study local hydrodynamic dispersion changes in different biofilm systems. In this approach, preferential flow paths other than local advection/dispersion changes were used to evaluate biofilm phase growth. Kildsgaard and Engesgaard (2002) also evaluated biofilm growth indirectly by conducting tracer experiments in a two dimensional sandbox and visualized the interactions between biofilm growth and flow field (Figure 2-7).
Figure 2-7: Flow chamber used by Kildsgaard and Engesgaard (2002) (reproduced with permission – Appendix K) to study biofilm development in porous media under two-dimensional flow field. Tracer experiments were conducted to visualize flow path of nutrients within the porous medium. Evolution of tracer plume was also used to reveal flow field around bioclogged (i.e. biofilm saturated) regions.

Imaging methods for direct visualization of biofilm in porous media systems have also been developed. Direct optical imaging by UV or visible light (Niemet and Selker, 2001), light microscopy (Dupin and McCarty, 2000; Kim and Fogler, 2000), dual-energy gamma radiation (Oostrom et al., 1998), magnetic resonance imaging (Seymour et al., 2004a), and X-ray microtomography (Davit et al., 2011) have been used to detect and assess biofilm characteristics in porous media. Light microscopy based visualization has been used to study biofilm structure and microbial cell interactions at pore-scales (Dupin and
McCarty, 2000; Kim and Fogler, 2000). For example, using a light microscope and image analysis programs, biofilm thickness has been measured in the laboratory (Hoskins et al., 1999). However, the irregular and non-flat shape of the porous media particles restricts the applicability of such optical techniques to visualize biofilms in porous media (Geesey and Mitchell, 2008). Electron microscopy techniques (e.g. SEM and TEM) have also been used to determine biofilm thickness on solid particles in porous media (Rinck-Pfeiffer et al., 2000; Hand et al., 2008). However, the destructive nature of sampling, as well as the time needed for sample preparation for analysis by electron microscopy, has made SEM and TEM visualization techniques inappropriate for real-time visualization of biofilm evolution in porous media (Jean et al., 2004).

Pitts and Stewart (2008) have introduced a method to noninvasively visualize biofilm growth in glass capillary tubes using confocal scanning laser microscopy (CSLM) combined with fluorescent labeling techniques. However, as working distance in objectives of high-resolution microscopy is limited, depth of observation is very limited in CSLM based on the dense, solid, and opaque nature of porous media.

X-ray tomography techniques have been used extensively in imaging the fluid flow and colloids transport in porous media (Wildenschild et al., 2005; Davit et al., 2011). However, the lack of information regarding the biofilm adsorption properties or absence of proper biofilm-labeling techniques has restricted the applicability of this advanced technique in investigating biofilms in porous structures. In addition, potential damage that can occur to biological systems during exposure to high energy radiation is another limiting factor in the applicability of high energy techniques in biofilm research.
Some promising results have been published in the recent years, which indicate the principal feasibility of nonoptical techniques such as ultrasound (Shemesh et al., 2007) or NMR (Hoskins et al., 1999; Seymour et al., 2004a,b; Metzger et al., 2009) in porous media biofilm studies. However, their application is currently limited due to their high expense, low resolution, and the noise exhibited by natural materials, such as natural loam, soils, or stone cores.

In recent years, bioluminescence exhibited by natural and engineered microorganisms has been used to visualize microbial processes in different environments (Burlage et al., 1990; Sanseverino et al., 1993; Flemming et al., 1994; Ripp et al., 2000; Uesugi et al., 2001; Oates et al., 2005; Sharp et al., 2005; Trögl et al., 2007). Instruments used to capture or measure bacterial bioluminescence include luminometers (King et al., 1990), scintillation counters (Neilson et al., 1999), x-ray autoradiography (Langridge et al., 1994), 35-mm film (Grant et al., 1991) and CCD imaging (Flemming et al., 1994; Silcock et al., 1992; Waterhouse et al., 1996; Uesugi et al., 2001). However, except for CCD cameras, all the other techniques require destructive sampling of porous media and thus, do not allow for continuous nonintrusive observation of microbial processes during an experiment. Considering the fact that hydrological relationships are very sensitive to invasive methods, CCD cameras provide an opportunity to study biofilm development in porous media.

The use of a CCD camera provides several benefits. The most important advantage of using CCD cameras in biofilm studies is their ability to quantify light intensities in a completely non-destructive manner. It is also a non-intrusive technique, allowing microbial processes to occur uninterrupted. Additionally, variable exposure lengths allow
for detection of low light intensities without considerable background noise. Therefore, the combination of bioluminescent reporter bacteria in conjunction with the use of a CCD camera for detection of low light intensities provides a unique technique in imaging microbial processes, including biofilm studies, and monitoring changes in both microbial and hydrologic properties in porous media systems.

Bacterial bioluminescence has been used to investigate biofilm development in a flat-plate flow chamber (Sharp et al., 2005) and translucent packed beds (Bozorg et al., 2012) to monitor biofilm development in porous media. Also, as a reporter gene, green fluorescent protein (GFP) has been expressed in natural and engineered microorganisms to detect bacterial colonies in porous media (Tombolini et al., 1997; Tresse et al., 1998). In addition, growth, fate and transport of microorganisms in natural environments have been monitored by cloning and expressing luminescence (lux) genes in a variety of host microbial species (Burlage et al., 1990; Shaw et al., 1992; Uesungi et al., 2001; Oates et al., 2005; Trögl et al., 2007).

2.6.2 Porous Media Flow Chambers in Biofilm Research

Different small, medium, and large scale porous media biofilm flow chambers have been used to investigate the effect of biofilm development on porous media geohydrological properties. Columns, ranging from a few millimeters to several meters in length and up to one meter in diameter, are the most commonly used chambers in bioclogging experiments. Fixed-bed column reactors filled with various types of porous media are commonly used to generate experimental data on coupled hydrological, geophysical, and geochemical processes to calibrate and examine different porous media reactive transport models (Brooks and Carroll, 2002; Pace et al., 2003). In addition, fixed-bed column
reactors have also been used to investigate bacterial cell transport as well as solute consumption or degradation by microorganisms in porous media (Murphy et al., 1997; Smith et al., 1985). Fixed-bed columns have served to relate transport of bacteria and colloids to geohydrological features of geological media (Fisk et al., 1999; Smith et al., 1985), and to obtain rates of biogeochemical transformations in porous media under dynamic flow conditions (Benner et al., 2002; Hansel et al., 2003).

Nonetheless, excluding capillary tubes which can be used to represent a single pore (Bakke et al., 2001), the thick and curved surface of most columns inhibit direct visualization of evolving processes taking place within columns, and thus, restricts the use of imaging systems in column experiments. Therefore, transparent microscopic flowcells with flat plates and high optical quality, have been designed and constructed to investigate fundamental processes of biofilm evolution in porous media (Cunningham et al., 1991, 1995; Sharp et al., 1999, 2005; Nambi et al., 2003; Ross et al., 2007; Willingham et al., 2008). These flow cells can be made of materials that contain certain patterns (e.g. etched glasses) or can be filled by a thin layer of porous media (e.g. soil, sand, or glass beads). According to the size and optical quality of these chambers and the enclosed porous media, direct visualization techniques can be utilized to monitor biofilm development.

2.7 Importance and Applications of Biofilms

Most bacteria found in natural, medical, and industrial processes persist as biofilms which indicates significant impact of biofilms on human health and productivity of different industrial processes. From a human perspective, biofilms were considered
detrimental for many years. Formation of biofilms (dental plaque) on teeth can lead to
dental caries. Also, biofilm formation on implants and prostheses, including heart valves,
contact lenses, and dental implants can result in serious infections. In a variety of
industrial and medical applications, biofilm can also cause serious problems. In food and
medical applications, biofilms and their metabolic reactions are sources of contamination
and infection, whilst in industrial processes, they can interfere with the intended
performance of processes equipment, a phenomenon known as biofouling.

Biofouling is a common issue in water treatment plants and filtration systems where
biofilm formation leads to bioclogging in filters and pipes. Permeability reduction due to
bioclogging has been identified in the field at the bottom of artificial water reservoirs
(Mirtskhulava et al., 1972), during the disposal of septic tank effluents (Kristiansen,
1981), or waste water disposal (Laak, 1970; McIntyre and Riha, 1991). Biofilm formation
in sand filters can negatively affect their performance in waste water treatment or
treatment of drinking water (Urfer et al., 1997). Also, bioclogging at the vicinity of the
discharge wells and the aquifer nearby can be troublesome during groundwater discharge
(Okubo and Matsumoto, 1979; Rinck-Pfeiffer et al., 2000; van Beek and van der Kooij,
1982).

In enhanced bioremediation of organic contaminants, nutrients and microorganisms are
injected to facilitate pollutants biodegradation. However, providing favorable conditions
for microbial growth not only enhance pollutants removal, but also increase probability
of bioclogging at the regions close to the injections wells where the optimal conditions
for microorganism present. Such bioclogging and permeability reduction in aquifer near
the injection wells will lower the capacity of the injection wells, whether by decreasing
the flow rates or increasing the injection pressure, and thus, reducing the overall efficiency of the bioremediation processes (Taylor and Jaffe, 1991; MacCarty et al., 1998). Detrimental biofilms grow inside pipes can reduce flow capacity in pipelines and increase friction and pressure loss (Figure 2-8A), which is a major problem in water distribution systems. Some biofilms contribute also to corrosion in metal pipes. In addition, the presence of biofilms on surfaces (Figure 2-8B) in cooling towers, and heat exchanges increase heat transfer resistance (Meesters et al., 2003).

Figure 2-8: A) *Pseudomonas fluorescense* biofilm formed inside a rectangular tube. Biofilm formation on the interior surface of pipes reduce the cross-sectional area and reduce flow capacity, increasing friction and head loss. They also can contribute to corrosion in metal pipes. B) *Pseudomonas fluorescens* biofilm formed on a piece of steel plate. Formation of biofilm on surfaces in heat exchangers or cooling towers results in corrosion and heat transfer reduction.
However, bioclogging, as an integral part of our life and the natural environment, can serve beneficial purposes. Bioclogging can be an important process on the field scale applications. There is strong evidence that bioclogging can reduce the heterogeneity of aquifers. In order to have high degradation rates, all nutrients, including contaminants and electron acceptors should be available for microorganisms at all time (Baveye et al., 1998). However, in a high heterogeneous reservoir with preferential flow paths, not only the actual residence time reduced (Benner et al., 2001), but also the regions with low permeability can be bypassed by the nutrients carried by the water, and thus, pollutants in the high contamination regions cannot be effectively removed. Therefore, heterogeneity of the aquifer and presence of preferential flow paths may reduce treatment efficiency in natural and engineered bioremediation processes (Lappan and Fogler, 1996; MacLeod et al., 1988). To reduce the negative impact of such heterogeneities, biofilm growth can be stimulated in high permeability regions to plug the preferential flow paths and establish a more homogenous flow field to extend the biologically active zones.

Subsurface permeable, semipermeable, or impermeable biobarriers can be developed by engineering biofilm structure and its growth pattern in an aquifer to control transport and/or remediate contaminants to preserve groundwater resources (Figure 2-9) (Komlos et al., 2004). Reactive biobarriers, which are also called biocurtains, can be built up by injection of nutrients into a reservoir to motivate growth of the indigenous microorganisms. In addition, microorganisms with special capabilities can be injected for specific reasons to enhance biological processes in the reservoir.
Figure 2-9: By engineering biofilm structure and its growth pattern, permeable and impermeable biobarriers can be developed which can be utilized to remove pollutants and control the migration of contaminants in an aquifer.

Another beneficial application of bioclogging is related to the water flooding of petroleum reservoirs, process referred to as microbial enhance oil recovery (MEOR). In the secondary oil recovery process, the oil reservoir is flushed by water in order to push the trapped oils toward the production wells. However, in reservoirs, oil is usually trapped in low permeable zones. Hence, during water flooding, the injected water flows through the preferential flow paths established by the regions of high permeability and may bypass the trapped oils encapsulated in low permeable zones. Such thief zones can result in poor sweep efficiency and consequently can negatively affect the oil production rates, water cut ratio, and ultimate recovery (Lappan and Fogler, 1994). To overcome such problems, as illustrated in Figure 2-10, impermeable biobarriers can be established
Figure 2-10: Application of biofilm growth and bioclogging in microbial enhanced oil recovery (MEOR). Biofilm growth and development of impermeable biobarriers enhances the amount of recovered oil by bioclogging the preferential flow paths with high permeability. Therefore, the injected water can go through the low permeable regions and sweep the encapsulated trapped oils and push them through the production wells.

Based on the applications that biofilms have and also the major impacts that bioclogging can have in different processes, it is important to study the processes leading to biofilm formation and also investigate the interactions exist between biofilm accumulation, amount of biomass, permeability variations, and porosity. Biofilm development has been studied in several experiments conducted in porous media flow chambers and interactions between biofilm growth and geohydrological properties have been investigated (Frankenberger et al., 1979; Okubo and Matsumoto, 1983; Taylor and Jaffé, 1990a;
Vandevivere and Baveye, 1992a,b; Seki et al., 1998; Holm, 2000; Bielefeldt et al., 2002a,b; Bozorg et al., 2012). Most of the studies investigating bioclogging of saturated porous media were conducted in laboratory column systems. Understanding interactions between biofilm evolution and porous media hydraulic properties would allow bioclogging to be avoided, where it has negative consequences, or promoted, where it has positive outcomes.

Here in this thesis, biofilm development and its influences on porous media geohydrological properties have been studied both theoretically and experimentally. A novel conceptual mathematical model was developed to simulate mutual interactions between biofilm evolution and porous media hydraulic properties. In addition, biofilm evolution in porous media was investigated in one and two-dimensional flow field, providing more detailed insight on dynamic biofilm behavior in porous structures.
Chapter 3

A New Approach to Model the Spatiotemporal Development of Biofilm Phase in Porous Media

3.1 Preface

This chapter is published as a manuscript in Environmental Microbiology journal, 2011, Volume 13, Pages 3010-3023 (DOI: 10.1111/j.1462-2920.2011.02578.x). A copy of the copyright permission from the publisher to republish the manuscript in this dissertation is provided in Appendix K. This manuscript is co-authored by I. D. Gates and A. Sen.
3.2 Abstract

Bacteria can exist within biofilms that are attached to the solid matrix of a porous medium. Under certain conditions, the biomass can fully occupy the pore space leading to reduced hydraulic conductivity and mass transport. Here, by treating biofilm as a growing, high viscosity phase, a novel macroscopic approach to model biofilm spatial expansion and its corresponding effects on porous medium hydraulic properties is presented. The separate yet coupled flow of the water and biofilm phases is handled by using relative permeability curves that allow for biofilm movement within the porous medium and bioclogging effects. Fluid flow is governed by Darcy’s law and component transport is set by the convection-diffusion equation reaction terms for each component. Here, the system of governing equations is solved by using a commercial multiphase flow reservoir simulator which is used to validate the model against published laboratory experiments. A comparison of the model and experimental observations reveal that the model provides a reasonable means to predict biomass development in the porous medium. The results reveal that coupled flow of water and movement of biofilm, as described by relative permeability curves, is complex and has a large impact on the development of biomass and consequent bioclogging in the porous medium.
3.3 Introduction

Under many conditions, planktonic bacteria attach to solid supports and proliferate to form bacterial films referred to as biofilms. Biofilms consist of cells embedded in a matrix of extracellular polysaccharide substances (EPS) secreted by the cells. Complex flow and diffusion pathways within the EPS matrix facilitate distribution of nutrients and removal of metabolic wastes as a biofilm evolves (Clement et al., 1996; Baveye et al., 1998).

Biofilm growth in a porous medium, and the consequent reduction of permeability, is important in water treatment, oil recovery, groundwater recharge, and in situ bioremediation (Thullner et al., 2002a, 2002b; Seifert and Engesgaard, 2007). The rate of biofilm growth is tied to the permeability and porosity distributions of the porous medium as well as the surface properties of the solid. As biomass spreads in a porous medium, the major controls on biofilm development, as displayed in Figure 3-1, include:

1. transport of nutrients, free suspended microbes and cell aggregates in the moving fluid,
2. attachment of microbes and cell aggregates to the solid matrix,
3. detachment of microbes and cell aggregates from the solid matrix, and
4. blocking of pores by filtration and cell growth.

Transport of entrained cell masses is controlled by the speed of flowing fluid, size of pore throats and bodies, and cell mass deformability which dictates their ability to “ooze” through pores. Fluid speed is controlled by matrix permeability, fluid viscosity, and pressure gradient. Rates of cell attachment to solid surfaces are controlled by pore sizes.
and cohesive forces between microbes. After cell masses attach, they grow and at some point, some fraction may detach from the main cell mass. Detachment is controlled by flow-induced shear forces and impact forces of other suspended cell masses or particles (Picioreanu et al., 2001). Detached biofilm fragments may become entrapped by downstream pore throats: filtration can occur due to bridging of pores. As pores become clogged, fluid flow through the remaining pore space falls. This reduces the volume of nutrient delivered to biomass located downstream of the blocked pore, thereby retarding further growth of biomass in that direction (Seki et al., 2006).

In order to engineer biofilms in porous media, models of biofilm attachment, detachment, colonization, growth and spreading are required. Here we report on a new biofilm transport and growth model based on treating biofilm as a viscous phase immiscible with the aqueous phase.

![Figure 3-1: Controlling activities affecting biofilm development in a porous medium.](image)
3.4 Background

Many experimental studies have investigated the impact of biofilm growth on porous media hydraulic properties (Seki et al., 1998, 2006; Kildsgaard and Engesgaard, 2001; Bielefeldt et al., 2002; Thullner et al., 2002a; Picireanu et al., 2004; VanGulck and Rowe, 2004; Arnon et al., 2005; Scheibe et al., 2007; Seifert and Engesgaard, 2007) and modeling of microbial transport and bioclogging in porous systems (Baveye and Valocchi, 1989; Taylor and Jaffe’, 1990; Tan et al., 1994; Clement et al., 1996; Noguera et al., 1999; Dupin et al., 2001; Kildsgaard and Engesgaard, 2001; Seki and Miyazaki, 2001; Thullner et al., 2002b, 2004; Kapellos et al., 2007).

Modeling approaches can be broadly divided in two categories. First, pore scale models are used to study biofilm behaviors and growth patterns at the level of the pores in order to determine their impact on hydraulic properties. These models attempt to link porosity to permeability (Dupin et al., 2001; Thullner et al., 2002b; Kapellos et al., 2007). In the early 1970s, mechanistically based biofilm modeling attempts began. These models were usually variants of simple steady one-dimensional (1D) mass transfer applied to first order kinetics theory to describe single species based fixed films (Chorin, 1967; Wilson and Geankoplis, 1966). These models, often too simple for design, provided a basis for future studies to obtain better understanding of fixed film processes (Harremoës, 1976; Harris and Hansford, 1976; Rittman and McCarty, 1980; Willson and McCarty, 1976). Even though the models provided some understanding of biofilm evolution, their predictions often deviated from experimental results. In the 1980s, 1D models were often still used, but some considered multiple species, mixed culture biofilms, and non-uniform distribution of the biomass types inside the biofilm (Kissel et al., 1984; Rittmann and
Manem, 1992; Wanner and Gujer, 1984; Wanner and Gujer, 1986). These models did not account for fluid flow in the pore space or within the biofilm, cell detachment, or shear forces on the biofilm. The transition to spatially-distributed transient models started largely in the 1990s. Many models included multiple active species, inert biomass, substrate utilization and diffusion within the biofilm, external mass transport, heterogeneous structure, and the effect of shear forces on both biofilm structure and detachment phenomena (Rittman and Manem, 1992). Some models used finite difference methods (Eberl et al., 2001; Laspidou and Rittmann, 2004; Noguera et al., 1999a,b; Picioreanu et al., 1998, 2001, 2004) or discrete methods (Noguera and Picioreanu, 2004; Pizarro et al., 2001), cellular automata (Laspidou and Rittmann, 2004; Noguera et al., 199b; Picioreanu et al., 1998; Pizarro et al., 2001), individual based (Kreft et al., 2001; Kreft and Wimpenny, 2001; Picioreanu et al., 2004), or continuum methods (Eberl et al., 2001). In some approaches, biofilm structures (surface shape, roughness, porosity) were modeled; these approaches generate porous biofilms with channels and voids within the biomass. Elber et al. (2001), by using a three-dimensional (3D) biofilm model that solved the full incompressible Navier-Stokes equations and mass transfer with nonlinear reactions in the biofilm, found that nutrient concentration, microbial growth rate, and initial biomass distribution were major controls on biofilm structure and its spatial heterogeneity. Picioreanu et al. (2004), by using 2D and 3D particle-based approaches to model the dynamics of multispecies biofilms growing on multiple substrates with spreading of biomass (represented by hard spherical particles), showed that structural variation and internal body forces caused by microbial growth.
Second, macro-scale models have been used to reproduce experimental data and to identify major processes involved in biofilm development and changes in conductivity. Often, biofilm is treated as a solid which can change the intrinsic porosity and permeability of the medium. In these models, biomass development follows a growth model tied to nutrient consumption (Eberl et al., 2000; Kildsgaard and Engesgaard, 2001; Thullner et al., 2004; Oates et al., 2005; Demaret et al., 2009). However, although macro-scale models can qualitatively reproduce experimental results, they are limited due to lack of reliable correlations between biofilm growth, porosity changes, and permeability modifications (Molz et al., 1986; Baveye and Valocchi, 1989; Clement et al., 1996, 1997; Baveye et al., 1998). To overcome these issues and reduce the uncertainty effects imposed by porosity/permeability correlations, we report here on a new biofilm transport and growth model based on treating biofilm as a viscous phase immiscible with the aqueous phase.

Recent reports assert that biofilms behave as viscoelastic fluids (Gujer and Wanner, 1990; Stoodley et al., 1999; Picioreanu et al., 2001; Dockery and Klapper, 2002; Klapper et al., 2002; Cogan and Keener, 2004; Cogan, 2008). As such, we have treated biofilm as a high viscosity phase which shares pore space with a low viscosity aqueous phase. In effect, we have modeled biofilm development in porous media as a multiphase flow process. This modeling framework helps to better understand biofilm behavior in porous media and can be used to predict how biomass might distribute in porous media under different environmental and operational conditions.
3.5 Materials and Methods

3.5.1 Model Formulation and Implementation

To engineer biofilms in porous media, models of biofilm attachment, detachment, colonization, growth and spreading will be required. In porous media, fluid flow obeys Darcy’s law and nutrient and waste transport are governed by the convective-diffusion equation. The new model is based on the following assumptions:

1. Water, porous medium matrix, and biofilm are treated as separate phases: the porous medium matrix is solid, water is a low viscosity phase and biofilm is a high viscosity phase. The fluid phases interact in the sense that pressure forces can move both phases but they move relative to each other according to their relative mobilities.

2. Interfacial tension between water and biofilm phases is negligible. This implies that there is no capillary pressure between the two phases.

3. All phases are incompressible.

4. Biofilm phase consists of microbial species and EPS.

5. Each microbial species consists of active and quiescent cell subpopulations. Note that quiescent cells include those that are not actively proliferating as well as dead ones.

6. Microbes can be convected in water phase.

7. Detachment and reattachment of microbes are represented by first order reactions that convert immobile microbes to mobile microbes or mobile microbes to immobile microbes.

8. There is a single growth-limiting nutrient that undergoes diffusive and convective mass transport in fluid phases.


10. The system is isothermal and the porous medium is isotropic.
3.5.2 Fluid Flow and Component Transport

The motion of fluid phases in a porous medium is given by Darcy’s law (Collins, 1961):

\[ \mathbf{q}_i = \mathbf{u}_i A = -\frac{k_{ri} k_{abs}}{\mu_i} A (\nabla P + \rho_i g) \tag{3.1} \]

where \( \mathbf{q}_i \) is the volumetric flow rate of phase \( i \), \( \mathbf{u}_i \) is the velocity of phase \( i \), \( A \) is the cross-sectional area, \( k_{ri} \) is the relative permeability of phase \( i \), \( k_{abs} \) is the absolute permeability of the porous medium, \( \mu_i \) is the viscosity of phase \( i \), \( P \) is the pressure, \( \nabla \) is the gradient operator, \( \rho_i \) is the density of phase \( i \), and \( g \) is the acceleration due to gravity. For the microbial phase, chemotaxis also sets a component of the transport velocity. Chemotaxis is the directed movement of bacteria in the direction of an attractant. However, Jang et al. (1982) and Sarkar et al. (1994) have shown that chemotactic movement is significant only under static conditions. Consequently, the water phase Darcy velocity is used as the flow velocity of planktonic bacteria.

For multiphase flow, the conservation of mass for water and biofilm phases together with Darcy's law yields:

\[ \nabla \cdot \left( \frac{k_{rw} k_{abs}}{\mu_w} \nabla P \right) = \frac{\partial}{\partial t} (\varphi S_w) + q_w \tag{3.2} \]

and

\[ \nabla \cdot \left( \frac{k_{rb} k_{abs}}{\mu_b} \nabla P \right) = \frac{\partial}{\partial t} (\varphi S_b) \tag{3.3} \]

where \( \varphi \) is porosity, \( S_w \) and \( S_b \) are the water and biofilm saturation (fraction of pore volume occupied by water and biofilm phases), respectively, and \( q_w \) is a volumetric
source or sink term for the water phase (only applies at a source or sink). Note that gravity effects are neglected since biofilm density is roughly equal to that of water (Ro and Neethling, 1991, Laspidou and Rittmann, 2004).

In the absence of biofilm ($S_b=0$), the water effective permeability equals the absolute permeability of the porous medium. As biofilm saturation rises, interference of the biofilm phase on water flow increases, and the water phase relative permeability drops. At the limit where biofilm saturation reaches unity, the water phase effective permeability drops to zero. At its residual saturation, the relative permeability of biofilm phase drops to zero (it becomes immobile). As biofilm grows, its saturation rises and its relative permeability and mobility increase. However, there are some restrictions for biofilm growth and mobility within porous structures. When biofilm saturation reaches unity (fully clogged pores), not only is there no water flow in the clogged pore space, but also further growth of the biofilm is prevented because of steric limitations and nutrient availability.

The transport of substrate species obeys Fick’s law, which after being inserted into the material balance, yields the convective-diffusion equation:

$$\nabla \cdot \left[ \left( \frac{k_{w} k_{ab}}{\mu_w} C_{wj} \nabla P + D_{wj} \nabla (\varphi S_w C_{wj}) \right) + \left( \frac{k_{rb} k_{ab}}{\mu_b} C_{bj} \nabla P + D_{bj} \nabla (\varphi S_b C_{bj}) \right) \right]$$

$$= \frac{\partial}{\partial t} \left[ \varphi \left( S_w C_{wj} + S_b C_{bj} \right) \right] + Q_w C_{wj} + R_{wj} + R_{bj} \quad (3.4)$$

where the left side represents convection and diffusion/dispersion mass transfer of component $j$ in water and biofilm phases. The right side terms correspond to mass accumulation, overall volumetric injection/production rate of water (includes both
background, \( Q_{w,B} \), and nutrient, \( Q_{w,N} \), and overall reaction rates of component \( j \) in each phase.

In Equation 3.5, the biofilm phase diffusion-dispersion term \((D_{bj})\) can be neglected since the diffusion rate of the viscous biofilm phase in pore space is small. For the water phase components, the diffusion-dispersion coefficient is given by:

\[
D_{wj,*} = \frac{D_{wj}^M}{\tau} + \alpha_{wj,*}|\mathbf{u}|
\]  

(3.5)

where the first term on the right side represents molecular diffusion of component \( j \) in the water phase and the tortuosity, \( \tau \), represents the deviation of actual flow from a straight path (Weissberg, 1963). The second term represents mechanical dispersion where \( \alpha_{wj,*} \) is the dispersivity of component \( j \) in the water phase in direction \( * \) (Bear, 1967).

Here, biofilm phase movement is controlled by its relative permeability. In some modeling attempts (Eberl et al., 2001; Kreft et al., 2001; Demaret et al., 2009), biofilm spreading occurs by diffusion/dispersion in the water phase. However, biofilm growth and expansion are coupled processes which cannot be fully captured by diffusion/dispersion because concentration gradient is not the only driving force for biofilm movement. Biofilm movement can occur by convection based on a self-generated pressure gradient that results from its growth. Biofilm relative permeability must have several characteristics. First, the biofilm saturation must reach a critical value before it can move. Second, the larger the biofilm saturation beyond this critical value, the more mobile it becomes until the porous medium becomes clogged. To satisfy these requirements, the biofilm relative permeability was assumed to follow a Gaussian function, shown in Figure 3-2, given by:
\[ k_{rb} = k_{rbmax} e^{-\frac{(s_b - S_{bmax})^2}{2\sigma^2}} \]  

(3.6)

where \( k_{rbmax} \) is the maximum relative permeability corresponding to a biofilm saturation of \( S_{bmax} \) and \( \sigma \) is an empirical parameter that controls the width of the curve. For water, the LET-type relative permeability model, named after its inventors Lomeland, Ebeltoft, and Thomas, also plotted in Figure 3-2, is used (Lomeland et al., 2005):

\[ k_{rw} = \frac{k_{rw0}(1-S_b)^L}{(1-S_b)^L + ES_b^T} \]  

(3.7)

where \( k_{rw0} \) is the maximum relative permeability of water phase and \( L, E, \) and \( T \) are empirical parameters.

Figure 3-2: Water and biofilm phase relative permeabilities versus biofilm phase saturation.
3.5.3 Reactions

The last term in Equation 3.5 represents biological and physicochemical (bacterial attachment and detachment) reaction rates for biological interaction of substrate concentration and bacterial growth. Here, we use first order reactions to represent biological and physicochemical behavior of bacteria in fluid and biofilm phases. It is assumed that bacteria duplicate when exposed to nutrient, and duplication is restricted to bacterial and nutrient concentrations according to the (unbalanced) reaction:

\[ \text{microbes} + \text{nutrient} \rightarrow \text{more microbes} \]  

(3.8)

Reaction 3.8 applies to both planktonic and attached biofilm bacteria. Also, the reaction indicates that when nutrients are depleted, the reaction rate equals zero. The reaction terms for planktonic and attached biofilm bacteria are respectively given by:

\[ R_{pb} = \lambda_{pb} S_w C_{pb} \]  

(3.9)

\[ R_{bb} = \lambda_{bb} S_b C_{bb} \]  

(3.10)

where \( R_{pb} \) and \( R_{bb} \), \( C_{pb} \) and \( C_{bb} \), and \( \lambda_{pb} \) and \( \lambda_{bb} \) are reaction terms, concentrations, and specific growth rates of planktonic and biofilm bacteria, respectively. Here, growth rates for planktonic and biofilm bacteria are the same:

\[ \lambda_{pb} = \lambda_{bb} = \lambda \]  

(3.11)

where \( \lambda \) is the specific growth rate. The Monod equation for the functional relationship between \( \lambda \) and limiting substrate concentration \( C_n \) is:
\[
\lambda = \frac{\lambda_{\text{max}} \varphi S_n C_n}{k_m + \varphi S_w C_n}
\]  
(3.12)

where \(\lambda_{\text{max}}\) is the maximum specific growth rate and \(k_m\) is the half maximum rate concentration of the limiting substrate.

The reaction governing microbial consumption of nutrient generates not only microbes but also EPS (Vandevivere and Baveye, 1992; Kreft and Wimpenny, 2001; Cogan and Keener, 2004). Here, EPS is produced by the microbes in the biofilm phase:

\[
\text{microbes} + \text{nutrient} \rightarrow \text{microbes} + \text{EPS}
\]  
(3.13)

Reaction 3.13 shows that EPS production requires both co-location of microbes and nutrient. Here, following Laspidou and Rittmann (2002), the EPS formation rate is proportional to the bacterial growth rate and a growth-associated coefficient that depends on the fraction of nutrient directed to produce EPS in the biofilm phase:

\[
R_{\text{EPS}} = Y_{\text{EPS}/C_{bb}} \left( \frac{\lambda_{\text{max}} \varphi S_n C_n}{k_m + \varphi S_w C_n} \right) \varphi S_b C_{bb}
\]  
(3.14)

where \(R_{\text{EPS}}\) is the EPS production rate and \(Y_{\text{EPS}/C_{bb}}\) is the growth-associated EPS production coefficient. Consequently, the overall substrate utilization rate is related stoichiometrically to cell growth and product formation by:

\[
R_m = - \frac{\lambda_{pb} \varphi S_n C_{pb} + (1 + Y_{\text{EPS}/C_{bb}}) \lambda_{bb} \varphi S_b C_{bb}}{Y_{b/S}}
\]  
(3.15)

where \(Y_{b/S}\) is the yield coefficient of biofilm phase formation which measures the mass of biofilm phase produced (including both bacteria and EPS) per unit of nutrient consumed.
Generally this yield factor is less than unity which implies that the substrate consumption rate is greater than new biofilm phase formation rate.

Microbial quiescence is controlled by the following first order reaction:

\[ \text{viable microbe} \rightarrow \text{quiescent microbe} \]  (3.16)

For Reaction 3.16, planktonic and biofilm microbial quiescence rates are given respectively by:

\[ R_{q-pb} = \lambda_q \varphi S_w C_{pb} \]  (3.17)
\[ R_{q-bb} = \lambda_q \varphi S_b C_{bb} \]  (3.18)

where \( \lambda_q \) is the bacterial quiescence coefficient which is taken to be the same for both planktonic and biofilm bacteria.

Attachment and detachment of microbes to and from the solid matrix are controlled by linear first order rates which depend on planktonic and biofilm microbial concentrations for attachment and detachment, respectively, as:

\[ R_{att} = -\lambda_{att} \varphi S_w C_{pb} \]  (3.19)
\[ R_{det} = \lambda_{det} \varphi S_b C_{bb} \]  (3.20)

where \( R_{att} \) and \( R_{det} \) are attachment and detachment rates and \( \lambda_{att} \) and \( \lambda_{det} \) are attachment and detachment coefficients for planktonic and biofilm bacteria. The rate equations reveal that the attachment rate depends on the planktonic microbe concentration in water whereas the detachment rate depends on the attached microbe concentration in biofilm.
Thus, the detachment rate is not considerable at initial stages of biofilm growth since the biofilm saturation is low.

In summary, the overall reaction rate expression for planktonic microbes is:

\[
R_{pb} = (\lambda_{pb} - \lambda_q - \lambda_{att}) \phi S_w C_{pb} + \lambda_{det} \phi S_b C_{bb} \tag{3.21}
\]

which represents bacterial duplication, quiescence, attachment, and detachment, respectively.

For biofilm, the overall biological and physicochemical reaction rates for microbes includes duplication, quiescence, detachment and attachment, and can be expressed as:

\[
R_{bb} = (\lambda_{bb} - \lambda_q - \lambda_{det}) \phi S_b C_{bb} + \lambda_{att} \phi S_w C_{pb} \tag{3.22}
\]

Although there is evidence that biomass density varies spatially within biofilms (Characklis and Marshall, 1990), here, it has been assumed to be constant (Dupin et al., 2001; Eberl and Efendiev, 2003).

### 3.5.4 Numerical Model

The model described above is a reactive multiphase flow problem in a porous medium. Here, we have used a commercial reservoir simulator (CMG, 2009) to model the biofilm system. The governing equations are discretized by using the finite volume approach. The domain is tessellated into a set of gridblocks. The discretized version of the governing equations (Equations 3.3 to 3.8) are solved on each gridblock. At each time step, Newton’s method is used to solve the set of nonlinear equations.
3.5.5 Model Validation: Two-Dimensional (2D) Experiments

Two different 2D laboratory experiments with different inoculating methods, each reported in the literature, were selected to validate the new model described above.

3.5.5.1 Partially Inoculated Sandbox

Kildsgaard and Engesgaard (2002) performed an experimental study to investigate clogging due to microbial growth in a 2D sandbox, displayed schematically in Figure 3-3 with properties and dimensions listed in Table 3-1. The sandbox, bounded by thin rectangular transparent Plexiglas sheets, was 30 cm wide by 44 cm high with a 1 cm gap between the sheets. The sandbox was packed with homogeneous sand with particle sizes

![Figure 3-3: Schematic of experimental apparatus used by Kildsgaard and Engesgaard (2002) - partially inoculated sandbox. The central 13 cm region (Inoculation Zone) was filled with sand that had been pre-inoculated with biofilm generating bacteria. A nutrient containing solution was injected into the sandbox at the bottom boundary (reproduced with permission – Appendix K).](image-url)
Table 3-1: Properties of the partially inoculated sandbox experiment (Kildsgaard and Engesgaard, 2002)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Unit</th>
<th>Value</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand box height</td>
<td>---</td>
<td>m</td>
<td>0.44</td>
<td>(1)</td>
</tr>
<tr>
<td>Sand box width</td>
<td>---</td>
<td>m</td>
<td>0.30</td>
<td>(1)</td>
</tr>
<tr>
<td>Sand box gap</td>
<td>---</td>
<td>m</td>
<td>0.01</td>
<td>(1)</td>
</tr>
<tr>
<td>Grain size range</td>
<td>---</td>
<td>m</td>
<td>((3 – 6) \times 10^{-4})</td>
<td>(1)</td>
</tr>
<tr>
<td>Porosity</td>
<td>(\varphi)</td>
<td>---</td>
<td>0.39</td>
<td>(1)</td>
</tr>
<tr>
<td>Inlet background water flow rate</td>
<td>(Q_{w,B})</td>
<td>(\text{m}^3/\text{day})</td>
<td>9.6x10^{-3}</td>
<td>(1)</td>
</tr>
<tr>
<td>Inlet nutrient flow rate</td>
<td>(Q_{w,N})</td>
<td>(\text{m}^3/\text{day})</td>
<td>7.2x10^{-4}</td>
<td>(1)</td>
</tr>
<tr>
<td>Inlet nutrient concentration</td>
<td>(C_{w,no})</td>
<td>(g/\text{m}^3)</td>
<td>9x10^{-4}</td>
<td>(1)</td>
</tr>
<tr>
<td>Maximum specific growth rate</td>
<td>(\lambda_{max})</td>
<td>((g/\text{biomass})/(g/\text{biomass}))</td>
<td>7.17</td>
<td>(1)</td>
</tr>
<tr>
<td>Yield coefficient of biomass production</td>
<td>(Y_{bi/s})</td>
<td>((g/\text{biomass})/(g/\text{nutrient}))</td>
<td>0.15</td>
<td>(1)</td>
</tr>
<tr>
<td>Yield coefficient of EPS formation</td>
<td>(Y_{EPS/C_{eb}})</td>
<td>((g/\text{biomass})/(g/\text{EPS}))</td>
<td>19</td>
<td>(2)</td>
</tr>
<tr>
<td>Half maximum rate concentration of nutrient</td>
<td>(k_m)</td>
<td>(\text{m}^3/\text{day})</td>
<td>1.2</td>
<td>(1)</td>
</tr>
<tr>
<td>Absolute permeability</td>
<td>(k_{abs})</td>
<td>(\text{mD})</td>
<td>1x10^{-5}</td>
<td>(1)</td>
</tr>
<tr>
<td>Vertical dispersivity</td>
<td>(\alpha_{vt})</td>
<td>m</td>
<td>7.7x10^{-4}</td>
<td>(1)</td>
</tr>
<tr>
<td>Horizontal dispersivity</td>
<td>(\alpha_{wt})</td>
<td>m</td>
<td>6x10^{-5}</td>
<td>(1)</td>
</tr>
<tr>
<td>Molecular diffusion coefficient</td>
<td>(D_w^M)</td>
<td>(\text{m}^2/\text{day})</td>
<td>9.4x10^{-5}</td>
<td>(3)</td>
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<tr>
<td>Water viscosity</td>
<td>(\mu_w)</td>
<td>(g/(\text{m/s}))</td>
<td>1</td>
<td>(3)</td>
</tr>
<tr>
<td>Tortuosity</td>
<td>(\tau)</td>
<td>---</td>
<td>1.47</td>
<td>(4)</td>
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<tr>
<td>Quiescence rate constant</td>
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<td>(1/\text{day})</td>
<td>0.05</td>
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<tr>
<td>Attachment coefficient</td>
<td>(\lambda_{att})</td>
<td>(1/\text{day})</td>
<td>0.06</td>
<td>(5)</td>
</tr>
<tr>
<td>Detachment coefficient</td>
<td>(\lambda_{det})</td>
<td>(1/\text{day})</td>
<td>0.08</td>
<td>(5)</td>
</tr>
<tr>
<td>Biofilm phase viscosity</td>
<td>(\mu_b)</td>
<td>(g/(\text{m/s}))</td>
<td>8000</td>
<td>(5)</td>
</tr>
<tr>
<td>Initial biomass saturation</td>
<td>(S_{bo})</td>
<td>(\text{m}^3/\text{biofilm}/\text{m}^3\text{pore space})</td>
<td>1x10^{-5}</td>
<td>(5)</td>
</tr>
</tbody>
</table>

(1) Kildsgaard and Engesgaard, 2002; (2) Thullner et al., 2002b; (3) Lide, 2006; (4) Weissberg, 1963; (5) Fitted
between 0.3 and 0.6 mm. For the clogging experiment, a 13 cm thick layer of inoculated sand was placed between two layers of sterile sand as shown in Figure 3-3. Degassed water was injected at the bottom boundary of the sandbox at 400 mL/h. Nutrient was injected at 30 mL/h through an injection port located upstream of the inoculation zone. At specific time intervals, a pulse of dye tracer was injected into the apparatus to visualize the effects of biofilm growth on the flow field in porous medium.

3.5.5.2 Fully Inoculated Sandbox

In another study, Thullner et al. (2002a) conducted a 2D experiment in a flow apparatus with dimensions 56 cm high and 44 cm wide with a gap equal to 1 cm. This apparatus is shown schematically in Figure 3-4 with key properties listed in Table 3-2. The gap,

![Figure 3-4: Schematic of experimental apparatus used by Thullner et al. (2004) - fully inoculated sandbox. The entire box was filled with sand that had been pre-inoculated with biofilm generating bacteria. A nutrient containing solution was injected into the sandbox at the bottom boundary (reproduced with permission – Appendix K)](image-url)
Table 3-2: Properties of the fully inoculated sandbox experiment (Thullner et al., 2004)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
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<th>Value</th>
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<tr>
<td>Sand box gap</td>
<td>---</td>
<td>m</td>
<td>0.01</td>
<td>(1)</td>
</tr>
<tr>
<td>Grain size range</td>
<td>---</td>
<td>m</td>
<td>(4 – 6) x 10^-4</td>
<td>(1)</td>
</tr>
<tr>
<td>Porosity</td>
<td>φ</td>
<td>---</td>
<td>0.38</td>
<td>(1)</td>
</tr>
<tr>
<td>Inlet background water flow rate</td>
<td>Q_{w,B}</td>
<td>m^3/day</td>
<td>2 x 10^-3</td>
<td>(1)</td>
</tr>
<tr>
<td>Inlet nutrient flow rate</td>
<td>Q_{w,N}</td>
<td>m^3/day</td>
<td>4.0 x 10^-5</td>
<td>(1)</td>
</tr>
<tr>
<td>Inlet nutrient concentration</td>
<td>C_{w,mo}</td>
<td>g/m^3</td>
<td>1 x 10^-3</td>
<td>(1)</td>
</tr>
<tr>
<td>Maximum specific growth rate</td>
<td>( \lambda_{\text{max}} )</td>
<td>(g biomass)/(g biomass)/day</td>
<td>0.8</td>
<td>(1)</td>
</tr>
<tr>
<td>Yield coefficient of biomass production</td>
<td>( Y_{b/s} )</td>
<td>(g biomass)/(g nutrient)</td>
<td>0.2</td>
<td>(1)</td>
</tr>
<tr>
<td>Yield coefficient of EPS formation</td>
<td>( Y_{E_{PS}/C_{b}} )</td>
<td>(g EPS)/(g biomass)</td>
<td>19</td>
<td>(1)</td>
</tr>
<tr>
<td>Half maximum rate concentration of nutrient</td>
<td>( k_{m} )</td>
<td>(g nutrient)/m</td>
<td>1.2</td>
<td>(1)</td>
</tr>
<tr>
<td>Absolute permeability</td>
<td>( k_{abs} )</td>
<td>mD</td>
<td>2.3 x 10^5</td>
<td>(1)</td>
</tr>
<tr>
<td>Vertical dispersivity</td>
<td>( \alpha_{vl} )</td>
<td>m</td>
<td>2 x 10^-3</td>
<td>(1)</td>
</tr>
<tr>
<td>Horizontal dispersivity</td>
<td>( \alpha_{vt} )</td>
<td>m</td>
<td>1 x 10^-4</td>
<td>(1)</td>
</tr>
<tr>
<td>Molecular diffusion coefficient</td>
<td>( D_{w}^{M} )</td>
<td>m^2/day</td>
<td>9.4 x 10^-5</td>
<td>(2)</td>
</tr>
<tr>
<td>Water viscosity</td>
<td>( \mu_{w} )</td>
<td>g</td>
<td>1</td>
<td>(2)</td>
</tr>
<tr>
<td>Tortuosity</td>
<td>( \tau )</td>
<td>---</td>
<td>1.48</td>
<td>(3)</td>
</tr>
<tr>
<td>Quiescence rate constant</td>
<td>( \lambda_{q} )</td>
<td>1/day</td>
<td>0.08</td>
<td>(1)</td>
</tr>
<tr>
<td>Attachment coefficient</td>
<td>( \lambda_{\text{att}} )</td>
<td>1/day</td>
<td>0.096</td>
<td>(1)</td>
</tr>
<tr>
<td>Detachment coefficient</td>
<td>( \lambda_{\text{det}} )</td>
<td>1/day</td>
<td>0.128</td>
<td>(1)</td>
</tr>
<tr>
<td>Biofilm phase viscosity</td>
<td>( \mu_{b} )</td>
<td>g</td>
<td>8000</td>
<td>(4)</td>
</tr>
<tr>
<td>Initial biomass saturation</td>
<td>( S_{bo} )</td>
<td>m^3/biofilm/m^3/pore space</td>
<td>1 x 10^-5</td>
<td>(4)</td>
</tr>
</tbody>
</table>

(1) Thullner et al., 2002b; (2) Lide, 2006; (3) Weissberg, 1963; (4) Fitted
sandwiched between transparent Plexiglas sheets, was filled with glass beads of diameter 0.4-0.6 mm. Nutrient were injected into the apparatus at 40 mL/day through a port located 10 cm above the bottom boundary. The pressures were measured at the inlet and outlet of the flow cell. Before nutrient injection, 2 liters of inoculum were injected into the apparatus and it was assumed that the porous medium was homogeneously inoculated. Similar to Kildsgaard and Engesgaard (2002), the flow field was visualized by injecting color dye upstream of the nutrient injection port to visualize biofilm growth. Also, biofilm growth was measured by using a digital camera and a light box behind the flow cell: dark sections indicated the presence of biofilm.

3.6 Results and Discussion

3.6.1 Comparison of New Model versus Experiments

Numerical models of the flow cells described above were constructed in the multiphase flow reservoir simulator. It was assumed that there were no variations in the direction of the gap between the plates; therefore, a 2D grid was used to model the flow domain. For the partially inoculated sandbox (Kildsgaard and Engesgaard, 2002), the domain was divided into 320,000 gridblocks (each one 0.7 mm wide by 0.5 mm high). In the fully inoculated sandbox (Thullner et al., 2002a), the domain was tessellated into 597,000 gridblocks (each one 0.7 mm wide by 0.5 mm high). For both models, constant flux conditions were applied at top and bottom boundaries. No flow was allowed at side boundaries. Nutrient and dye were injected at constant flow rates. Since initial microbial concentrations were not reported by either research group, the initial saturations of the biofilm phases in each experiment were taken to be $1 \times 10^{-5} \text{ (m}^3\text{ Biofilm)/(m}^3\text{ Porespace)}$. 

67
Initially, the biofilm phase contained both immobile cells and EPS. During injection, all injected fluids were taken to be free of the microbial phase.

The parameters that govern the relative permeability of the biofilm phase, listed in Equation 3.7, were adjusted until the biofilm distribution of the model was similar to that of the experiments, as indicated by the flow field of the dye. Thus, the criterion under which we have made the comparison is by visual appearance alone of the tracer plume within the flow cell. Starting with identical initial conditions, the results from the modeling studies were found to correlate well with the experimental studies. In both cases the fluid flow paths were found to change in a similar manner over time, thereby suggesting comparable local hydraulic permeability changes due to biofilm development. Table 3-3 lists model parameter values which give the best agreement with experimental observations.

Table 3-3: Parameters used for the water and biofilm phase relative permeability functions (Equation 3.6 and 3.7) in order to simulate 2D biofilm experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>2.5</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
</tr>
<tr>
<td>T</td>
<td>1.5</td>
</tr>
<tr>
<td>$k_{rw}^0$</td>
<td>1</td>
</tr>
<tr>
<td>$k_{rbmax}$</td>
<td>0.32</td>
</tr>
<tr>
<td>$S_{bmax}$</td>
<td>0.65</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>0.06</td>
</tr>
</tbody>
</table>
3.6.1.1 Partially inoculated sandbox

Figure 3-5 compares images of the flow field over time as marked by the injected dye in the experiments (Figure 3-5A) versus continuous tracer injection in our new simulation model (Figure 3-5B). The tracer used in the simulation model consists of a water component with the same properties as the aqueous phase. The results demonstrate that the model provides a reasonable representation of the growth of the biofilm phase in the porous medium. Kildsgaard and Engesgaard (2002) injected pulses of dye, and thus, tracer plumes observed in their experiments are shorter than in the model results. Both experimental observations and model results show that the tracer plume splits when it encounters biofilm within the porous medium. This flow pattern reveals not only how the biofilm is growing in the domain, but that the growth leads to clogging of the porous medium, thereby forcing aqueous phase to move around the biofilm.

The bypass of flow implies that the core of the biofilm will experience reduced nutrient delivery. Also, both the experiment and model reveal that biofilm phase migrates towards the nutrient injection site as the system evolves through time. A comparison of the shape of the tracer plumes observed experimentally and that obtained from the model reveals some differences: the model results are symmetrical whereas this is not observed in the experiments. This could occur if the initial microbial concentration was not uniform in the inoculated zone of the experiment.
Figure 3-5: Flow field around biofilm phase at different time intervals as marked by tracer injection (shown in dark black). Shown are (A) experimental results from Kildgaard and Engesgaard (2002) (reproduced with permission) and; (B) results obtained from the new model. Tracer flow patterns reveal regions clogged due to biofilm growth and development. Area between the dotted lines shows the 13 cm inoculated region.
The biofilm saturation over time from the model is displayed in Figure 3-6. The images reveal that the biofilm first grows along the trajectory of nutrient flow within the inoculation zone. As the biofilm phase grows, it tends to move towards the source of the nutrients, that is, towards the nutrient injection port. Up to ~10 days, the biofilm phase remains as a strip with constant width within the inoculation zone. A comparison of the results displayed in Figures 3-5 and 3-6 indicates that at times less than 6 days, the permeability is largely unchanged due to low biofilm phase saturation. After 10 days, the biofilm saturation exceeds 0.60 and its areal extent is large enough to impact overall liquid flow patterns. Due to its reduced relative permeability, the nutrient-carrying aqueous phase cannot easily penetrate into regions of high biofilm saturation.

Figure 3-6: Predictions of the biofilm phase saturation over time in Kildsgaard and Engesgaard’s (2002) experiment using the new model. The area between the two dotted lines represents the inoculation region where microbes were uniformly inoculated at the beginning of the simulation. Region A represents the downstream biofilm finger. Area between the dotted lines shows the 13 cm inoculated region.
The corresponding drop in nutrient delivery to this region has two effects. First, biomass production occurs primarily at the periphery of the biofilm region and not within the biofilm itself. Second, microbial quiescence occurs more frequently than microbial growth in the interior of the biofilm region. As noted previously, microbial quiescence does not change hydraulic properties since quiescent cells are immobile and have the same properties as the biofilm phase. The streamlined flow of nutrient-carrying water that separates around the biofilm region leads to a relatively thin biofilm finger downstream of the inoculation zone. Detached biofilm cells from the upstream region are entrained by the flow around the clogged biofilm zone, and when the flows merge at the downstream end of the biomass, the cells can attach in this new region leading to the finger that extends in the flow direction (shown as Region A in Figure 3-6).

Figure 3-7 displays the water relative permeability distribution at Day 30, given by:

\[
k_{rw} = -\frac{q_w \mu_w}{A k_{abs} \nabla P} = -\frac{u_w \mu_w}{k_{abs} \nabla P}
\]  

(3.23)

As expected, it correlates with the biofilm distribution shown in Figure 3-6. The overall effective water permeability \((k_{rw}, k_{abs})\) decreases over time with biofilm evolution. The lowest values of relative permeability are at the upstream region of the biofilm phase where its saturation and growth rate are highest (shown as Region A in Figure 3-7). The relative permeability of water phase in this location is almost equal to zero which implies that the porous medium is clogged. Downstream of this region (labeled as Region B in Figure 3-7), the porous medium can be characterized as being partially clogged based on the lower degree of biofilm saturation. Thus, aqueous phase can still penetrate into this region, albeit at a relatively low rate, causing further biofilm growth and increased
clogging. At the centre of Region B, the relative permeability does not change significantly once it reaches 60% of the original value. This is likely due to lack of nutrient penetration into this region as a result of the clogged upstream section.

Figure 3-7: Prediction of relative permeability distribution of water phase after 30 days in Kildsgaard and Engesgaard’s (2002) experimental configuration. Region A represents the upstream section of the biofilm phase. Region B marks the downstream region of the biofilm phase whereas Region C embodies the downstream biofilm finger. Area between the dotted lines shows the 13 cm inoculated region.
Figure 3-8 shows the biofilm phase saturation at the end of the simulation (note, scale is different from that in Figure 3-6) and the evolution of the biofilm phase effective permeability versus time at five points. Biofilm saturation changes by two processes: biofilm growth and microbial detachment. At Point A, located downstream of the inoculation zone, biofilm growth is due to detachment and reattachment of cells from an upstream region. After 23 days, the biofilm saturation and its effective permeability at Point A become significant. The oscillatory behavior of the biofilm effective permeability after this time point can be explained by a simultaneous growth and detachment processes. High nutrient availability at this point promotes biofilm growth which leads to high biofilm saturation in a short time interval. According to Figure 3-2, biofilm phase permeability and saturation increases until a critical value of 65% is reached, beyond which, higher saturation leads to more severe clogging. Lack of nutrients in clogged pores reduces the biofilm growth rate. Eventually biofilm growth and development will stop when the pore is completely clogged. As shown in Equation 3.20, high biofilm saturation in clogged sections indicates high detachment rates, which in turn, results in reduced biofilm saturation. Reduced biofilm saturation means more water flow and nutrient availability, which leads to another increase in biofilm growth and saturation (i.e. clogging). This cycle of clogging and declogging (based on growth and detachment processes) leads to oscillatory behavior of the biofilm permeability at Point A. Point B is positioned upstream of the inoculation zone. At Day 14, the biofilm effective permeability spikes at this point but declines within 5 days to a small value. This behavior can be explained by high nutrient availability in this region which leads to rapid biofilm saturation (up to 70%). Due to high nutrient availability and biofilm growth
rate, the upstream section of Point B is rapidly clogged and consequently, no nutrient is accessible for biofilm located at Point B. This means no biofilm growth and development which has been predicted by almost zero biofilm permeability at this point after 20 days. Point C is located near the upper edge of the inoculation zones. The results reveal that biofilm effective permeability at this location exhibits two peaks during the simulation. Biofilm permeability changes at this point can be explained similar to Point A. High water saturation and nutrient availability at Point C causes biofilm growth followed by detachment. This results in oscillations of the biofilm effective permeability. According to Equation 3.20, the higher the biofilm saturation, the greater the rate of cell detachment, which leads to reduced biofilm saturation and consequently higher water
flow and nutrient availability. In clogged pores, this process encourages biofilm growth and consequently elevates biofilm permeability. At Point D, after 9 days the biofilm saturation reaches 65% which corresponds to the highest permeability of the biofilm phase (Figure 3-2). Further growth at Point D causes permeability reduction which drops to almost zero after 12 days. Even with low permeability, water can still enter partially clogged regions at low rate supplying a small amount of nutrient to sustain biofilm growth, attachment, and detachment. The results show that continuous penetration of small amounts of water (with nutrient) to the semi-clogged regions leads to continuous detachment of cells from Point D which in turn leads to saturation reduction and slight increase in water effective permeability. The effective permeability at Point E, located at the top of the initially inoculated zone, exhibits a peak at about Day 25, similar to that of Point A.

3.6.1.2 Fully inoculated sandbox

Figures 3-9 and 3-10 compare Thullner et al.’s (2002a) experimental observations and model predictions of biomass and injected tracer dye distributions with predictions of the model developed here near the nutrient injection port (8 cm by 11 cm observation zone). Model predictions for biofilm saturation near the nutrient injection port (Figure 3-9C) strongly support the approach used to simulate bioclogging processes. The results show continued increase of biomass saturation up to approximately 37% after 30 days of development. Also, the model predicts that the biofilm saturation is more significant at the edges of the biofilm plume and is less saturated in the central regions which compares
Figure 3-9: Biofilm distribution near nutrient injection port: (A) Experimental results from Thullner and colleagues (2004) (reproduced with permission) where the biofilm saturated regions are visible by a light bank behind the sandbox (darker gray level corresponds to higher biofilm saturation), (B) Mathematical model predictions from Thullner and colleagues (2004) (reproduced with permission), and (C) predictions from the model developed here. Gray scale shows biofilm phase saturation.

well with experimental observations (Figure 3-9A). Biofilm formation starts near the nutrient injection port and over time, forms a biofilm stripe with the same width and direction as the nutrient flow path. Also, as seen in Figure 3-10, the spatial distribution of the nutrient does not change much, which indicates that the permeability of water has not been altered significantly and biofilm saturation is not considerable. Based on the simulation results, the maximum biofilm saturation in this experiment is about 37% and it
Figure 3-10: Tracer distribution near nutrient injection port: (A) experimental results from Thullner and colleagues (2004) (reproduced with permission) where dark plume represents color tracer travels in porous medium and bypasses the bioclogged regions (darker gray level corresponds to higher tracer concentration), (B) mathematical model predictions from Thullner and colleagues (2004) (reproduced with permission), and (C) predictions from model developed here. Gray scales represent relative tracer concentration (i.e. tracer concentration / initial tracer concentration at tracer injection port).

does not significantly affect tracer transport which is in accordance with Figure 3-2 that shows a minimum water relative permeability of about 60%. Thus, nutrients can travel through these sections and biofilm growth occurs along the nutrient pathway. The results
also show that the tracer plume tends to split into two branches when it approaches the
nutrient injection port where the biofilm has grown and that these two branches merge
downstream of the nutrient injection port. The effect of biofilm on the tracer plume is
clear at the nutrient injection port which represents the edge of the biofilm phase.

3.6.2 Effects of Relative Permeability Curves

The results obtained from the model are impacted by the water and biofilm relative
permeability curves used (Equations 3.6 and 3.7). Since these curves are functions of
fitted parameters that are described in Table 3-3, the model results are highly dependent
on the values chosen to generate the relative permeability curves. As such, it is important
to determine the level of sensitivity that the model exhibits in response to changing
permeability values.

Table 3-4: Dimensionless parameters used in relative permeability functions
(Equations 3.6 and 3.7) to simulate and investigate the effect of relative
permeabilities on the overall biofilm phase development.

<table>
<thead>
<tr>
<th>Case</th>
<th>Description</th>
<th>$k_{rw}^0$</th>
<th>$L$</th>
<th>$E$</th>
<th>$T$</th>
<th>$k_{r_{b max}}$</th>
<th>$S_{b_{max}}$</th>
<th>$\sigma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>Fitted parameters to simulate experiments</td>
<td>1</td>
<td>2.5</td>
<td>1</td>
<td>1.5</td>
<td>0.32</td>
<td>0.65</td>
<td>0.06</td>
</tr>
<tr>
<td>(B)</td>
<td>Higher water relative permeability</td>
<td>1</td>
<td>1.5</td>
<td>0.5</td>
<td>4</td>
<td>0.32</td>
<td>0.65</td>
<td>0.06</td>
</tr>
<tr>
<td>(C)</td>
<td>Lower water relative permeability</td>
<td>0.7</td>
<td>2.5</td>
<td>1</td>
<td>1.5</td>
<td>0.32</td>
<td>0.65</td>
<td>0.06</td>
</tr>
<tr>
<td>(D)</td>
<td>Lower biofilm phase relative permeability</td>
<td>1</td>
<td>2.5</td>
<td>1</td>
<td>1.5</td>
<td>0.24</td>
<td>0.65</td>
<td>0.06</td>
</tr>
<tr>
<td>(E)</td>
<td>High biofilm phase relative permeability</td>
<td>1</td>
<td>2.5</td>
<td>1</td>
<td>1.5</td>
<td>0.42</td>
<td>0.65</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Figure 3-11 displays the impact of changing water and biofilm relative permeability curves on biofilm saturation for Kildsgaard and Engesgaard’s (2002) experimental configuration. Table 3-4 lists parameters used in five different cases: (A) control base case; (B) water relative permeability curve is raised compared to base case; (C) water relative permeability is lowered; (D) biofilm relative permeability is lowered; (E) biofilm relative permeability is raised. The results demonstrate that the spatial development of biofilm phase is sensitive to the relative permeability curves of both water and biofilm phases.

Figure 3-11: Model predictions of the biofilm development at the end of Kildsgaard and Engesgaard’s (2002) experiment (Day 30) for different relative permeability functions: (A) calibrated parameters (listed in Table 3-2), (B) higher water relative permeability values (L=1, E=1.5, T=4), (C) lower water relative permeability values (k_{rw}=0.7), (D) lower biofilm phase relative permeability values (k_{rbmax}=0.24), and (E) higher biofilm phase relative permeability values (k_{rbmax}=0.42). Gray scale shows biofilm phase saturation. Area between the dotted lines shows the 13 cm inoculated region.
Figure 3-11B reveals that larger water relative permeability increases biofilm phase saturation and size whereas Figure 3-11C shows that lower water phase relative permeability reduces the biofilm phase size. Higher water permeabilities indicate higher water mobility, which implies increased nutrient transport causing more biofilm growth. Also, higher water permeabilities mean greater water flow rates, which in turn imply more detachment of biofilm from the central biomass. This would lead to increased spreading of biofilm, especially in the downstream direction (see Figure 3-11B). Lower water relative permeability means smaller water flow rates which in turn leads to less nutrient transport and detachment leading to lower growth and less biofilm spreading, respectively. The lower the biofilm relative permeability, the less the spread of the biofilm phase, especially in the upstream direction.

These results show that the spatial development of biofilm phase predicted by the model, and the consequent development of bioclogged areas, and thus overall effective permeability of the system, is greatly impacted by the relative permeability curves chosen.
3.7 Conclusions

A new approach to model biofilm growth and spread in porous medium was developed by treating biofilm as a high viscosity phase that interacts hydraulically with the water phase. The model used Darcy’s law to deal with fluid flow and the convective-diffusion/dispersion equation to handle component transport. Importantly, relative permeability curves were used to couple the two phases instead of relying on explicit correlations between biofilm saturation, porosity, and permeability. The simulation results compared well against two-dimensional experimental observations and the relative permeability concept permitted modeling of water-channeling and water-rich zones within the biofilm mass. Occupation of an increasing fraction of the pore space by the biofilm was found to eventually drop the water relative permeability to zero, thereby resulting in bioclogging. The results also revealed that coupled flow of water and movement of biofilm, as described by the relative permeability curves, is complex and has a large impact on the development of the biomass and consequent bioclogging in the porous medium. The importance of biofilm and water relative permeability curves implies that more investigation is required to better understand the relationship between these phases in porous media.
Chapter 4

Real Time Monitoring of Biofilm Development

Under Flow Conditions in Porous Media

4.1 Preface

This chapter is published as a manuscript in the journal Biofouling, 2012, Volume 28, Pages 937-951 (DOI: 10.1080/08927014.2012.723204). A copy of the copyright permission from the publisher to republish the manuscript in this dissertation is provided in Appendix K. This manuscript is co-authored by I. D. Gates and A. Sen.

The present thesis is based on refereed papers that have already been published, or are in the process of publication. Unavoidably, there is some repetition between the chapters, particularly in the Introduction to each chapter. Therefore, in this chapter, sections of the Introduction may be safely skipped without loss of coherence. In addition, more comprehensive details of experimental procedures used to prepare culture media, sample the porous media, prepare biofilm samples, and determine bacterial population, as well as the assays used to measure bacterial DNA and protein concentration are provided in Appendices A through G. Also, MATLAB codes used to process the bioluminescence images are summarized in Appendix J.
4.2 Abstract

Biofilm growth can impact the effectiveness of industrial processes that involve porous media. To better understand and characterize how biofilms develop and affect hydraulic properties in porous media, both spatial and temporal development of biofilms under flow conditions was investigated in a translucent porous medium by using *Pseudomonas fluorescens* HK44, a bacterial strain genetically engineered to luminesce in the presence of an induction agent. Real-time visualization of luminescent biofilm growth patterns under constant pressure conditions was captured using a CCD camera. Images obtained over 8 days revealed that variations of bioluminescence intensity could be correlated to biofilm cell density and hydraulic conductivity. We used these results to develop a real-time imaging method to study dynamic behavior of biofilm evolution in a porous medium, thereby providing a new tool to investigate the impact of biological fouling in porous media under flow conditions.
4.3 Introduction

Biofilms are composed of dynamic communities of microbial cells enclosed in structured, self-produced matrices of hydrated extracellular polymeric substances (EPS) that adhere to inert or living surfaces (Costerton, 1995). It is believed that more than 90% of all bacteria live within biofilms (Characklis et al., 1990). The stages that lead to the development of a mature biofilm, including attachment of planktonic bacterial cells to a solid surface, colonization, growth, and EPS production, have been well investigated (Costerton, 1995; Costerton and Lappin-Scott, 1995).

Biofilms are encountered frequently in medical applications, as well as industrial and environmental processes. Biofouling of porous structures such as filters, soils, and petroleum reservoirs by biofilm can reduce the size of pores over time. This in turn can impact permeability of the porous medium, a phenomenon known as bioclogging. For example, bioclogging has been found to adversely affect the performance of sand filters used to treat wastewater (Nicolella et al., 2000) and drinking water (Urfer and Huck, 2001; Sharp et al., 2001). Biofouling in porous media leads to reduced hydraulic conductivity, and thus, a full understanding of interactions between microbes and their environment is critical to better design, operate, and control these systems (Thomas and Ward, 1989; Clement, 1996; Singh et al., 2006; Bishop, 2007).

The relationships between biofilm distribution in a porous medium, hydraulic conductivity and consequent impact on metabolic reactions are not fully understood. In most studies, biofilm growth is detected indirectly by monitoring the pressure drop (at fixed flow rate) across a system, or the flow rate (at fixed pressure drop) through the system (Taylor and Jaffè, 1990a,b; Kildsgaard and Engesgaard, 2002; Seki et al., 2006).
Also, most studies destructively sample the porous medium at the end of the experiment to measure biofilm cell density (Komlos et al., 2004; Seki et al., 2006). Here, we report on a method to simultaneously visualize biofilm development and evaluate cell density, hydraulic conductivity, and fraction of void space occupied by biofilm (i.e. biofilm saturation).

4.4 Background

At present, imaging methods to visualize the growth and impact of biofilm in porous media systems include direct optical imaging by UV or visible light (Niemet and Selker, 2001; Huang et al., 2002), dual-energy gamma radiation (Oostrom et al., 1998), magnetic resonance imaging (Seymour et al., 2004), and X-ray microtomography (Davit et al., 2011). In addition, many studies have demonstrated the effect of biofilm on hydraulic conductivity by using standard soil methods (Vandevivere and Baveye, 1992; Cunningham et al. 1991, 1997), dye tracer experiments (Thullner et al., 2002; Kildsgaard and Engesgaard, 2002; Seki et al., 2006), breakthrough curves (Wollum and Cassel, 1978; Harvey et al., 1989; Sharp et al., 1999), light microscopy (Dupin and McCarty, 2000; Kim and Fogler, 2000), and mathematical models (Vandevivere et al., 1995; Clement et al., 1996; Eberl et al., 2000; Thullner et al., 2004; Bozorg et al., 2011). Light microscopy based visualization has been used to investigate biofilm structure and cell interactions at pore-scale (Dupin and McCarty, 2000; Kim and Fogler, 2000). Electron microscopy techniques (e.g. SEM and TEM) have also been used to determine biofilm thickness on solid particles in porous media (Rinck-Pfeiffer et al., 2000; Hand et al., 2008). However, the destructive nature of sampling, as well as the time needed to prepare the samples for analysis by electron microscopy, makes SEM and TEM visualization
techniques inappropriate for real-time visualization of biofilm development in porous media.

In recent years, bioluminescence exhibited by natural and engineered microorganisms has been used to monitor microbial processes (Burlage et al., 1990; Sanseverino et al., 1993; Flemming et al., 1994; Ripp et al., 2000; Uesugi et al., 2001; Sharp et al., 2005). Sharp and coworkers (2005) used bioluminescent bacteria in an etched glass flat-plate flow chamber to study biofilm growth in a pore network. In this experiment, bacterial bioluminescence was used to track biofilm development, but no quantification was made in terms of detected bioluminescence intensities. Also, green fluorescent protein (GFP) has been expressed in bacterial cells as a reporter gene to detect bacterial colonies (Chalfie et al., 1994; Tombolini et al., 1997; Tresse et al., 1998). Constitutive production of GFP makes the gfp-gene an excellent marker to detect bacteria in complex substrata such as soil. Moreover, growth, fate and transport of microorganisms in natural environments has been monitored by cloning and expressing luminescence (lux) genes in a variety of host microbial species (Burlage et al., 1990; Shaw et al. 1992; Uesungi et al., 2001; Oates et al., 2005; Trögl et al., 2007). However, experimental methods described in the literature involving bioluminescent microorganisms can result in inconsistent induction of lux genes due to issues related to the mass transfer of induction agents. Here, we describe a method to address these mass transfer issues, thereby allowing consistent bioluminescence of microbial species containing inducible lux genes in porous media. Using this method, we have been able to nondestructively observe how biofilms grow and disperse in a porous medium while simultaneously measuring the hydraulic conductivity.
4.5 Materials and Methods

4.5.1 Bacterial Strain

The bacterial strain used here was the bioluminescent reporter strain *Pseudomonas fluorescens* HK44 obtained from the University of Tennessee Center for Environmental Technology (Knoxville, Tennessee). HK44 is a rod-shaped, gram-negative bacterium originally isolated from soil heavily contaminated with polyaromatic hydrocarbons and heavy metals (Sanseverino 1993). It carries the naphthalene catabolic plasmid pUTK21 and was genetically modified by transposon insertion of the salicylate-inducible nahG-luxCDABE (bioluminescent) gene cassette and a tetracycline resistance marker (King et al., 1990). This strain emits luminescent light in the presence of naphthalene, salicylate, 4-methyl salicylate, and other aromatic hydrocarbons (Sanseverino et al., 1993; Ripp et al., 2000).

4.5.2 Media

Three different media were used: one to maintain and expand cell populations in planktonic culture, a second one for flow chamber studies, and a third medium to induce bioluminescence for visualization. The pH of each medium was adjusted to 7.2 ± 0.05 with 1 M NaOH or HCl and all media were autoclaved at 121°C for 20 minutes prior to being used. Stock solutions were stored at 4°C for up to two weeks. Also, to ensure plasmid maintenance, all media used were amended with 30 mg/L (final concentration) tetracycline (EMD Chemicals, OmniPur® EM-8990). Tetracycline solution was prepared in 50% ethanol (v/v with water) and added through a 0.2 µm filter to the autoclaved media.
4.5.2.1 Stock Culture Growth Medium

Oxygen-saturated nitrate-free growth medium was used to maintain a stock of cells for use in this study. All cell stocks were generated in a shaken, batch culture vessel where they maintained a planktonic phenotype. One liter of growth medium was generated by combining (please see Appendix A for more details):

(i) 333.3 mL of mineral based medium consisting of MgSO$_4$ (1.2 g/L), CaCl$_2$.2H$_2$O (0.3 g/L), NH$_4$Cl (1.2 g/L),

(ii) 332.4 mL of phosphate buffer saline (PBS) solution consisting of NaCl (24 g/L), KCl (0.6 g/L), NaH$_2$PO$_4$ (3.45 g/L), K$_2$HPO$_4$ (0.78 g/L),

(iii) 1.0 mL of trace element solution containing HCl (3.66 g/L), FeSO$_4$.7H$_2$O (21 g/L), H$_3$BO$_3$ (0.3 g/L), MnCl$_2$.4H$_2$O (1 g/L), CoCl$_2$.6H$_2$O (1.9 g/L), NiCl$_2$.6H$_2$O (0.24 g/L), CuCl$_2$.2H$_2$O (0.02 g/L), Na$_2$EDTA.2H$_2$O (10 g/L), ZnSO$_4$.7H$_2$O (1.44 g/L), Na$_2$MoO$_4$.2H$_2$O (0.36 g/L), and

(iv) 333.3 mL of glucose solution (3 g/L) as the main carbon source.

4.5.2.2 Medium for Biofilm Growth in Flow Chamber

The growth medium described above for stock culture was also used in all experiments carried out in the porous medium flow chamber. However, when used in the flow chamber, the glucose concentration was lowered to 0.25 g/L; high concentrations were not required since the spent medium was continuously replenished with fresh medium containing glucose (unlike in the batch cultures). For clarity, this medium will be referred to as biofilm growth medium.
4.5.2.3 Induction Medium

Induction medium was generated by using the same components listed for the stock culture growth medium, with the exception of phosphate sources. The elimination of phosphate from the medium allowed available nutrients to be dedicated to bioluminescence, and minimized other cell activities including cell division. The medium was also supplemented with 0.1 g/L (final concentration) of salicylate to induce bioluminescence. Continuous bioluminescence requires a considerable amount of cellular energy, with ATP levels in bacterial cells dropping by as much as an order of magnitude (DeLuca 1978). In addition, our preliminary results indicated that low oxygen concentrations negatively affect HK44 bioluminescence levels. Thus, to have constant and continuous bioluminescence, the induction medium was supplemented with 1.0 g/L glucose (instead of 0.25 g/L in the flow chamber medium used for biofilm development in the porous medium) and fully saturated with oxygen prior to injection into the chamber.

4.5.3 Chamber Inoculation

A stock culture of bacterial strain HK44 (0.1% v/v) was prepared at room temperature in oxygen-saturated growth medium (please see Appendix A for more details). Following overnight incubation (shaken at 150 rpm) at room temperature, 0.1 mL of the culture (4.13x10^8 cells/mL) was inoculated into 30 mL of fresh growth medium. After 24 hours, cells were harvested by centrifugation (Beckman Coulter®, X-22R) at 5000 rcf for 20 min, washed in PBS, centrifuged again and resuspended in growth medium to 6x10^6 cells/mL. This culture was used as the flow chamber inoculum. All population densities
were determined by measuring light absorbance at 550 nm (Appendix E) in a spectrophotometer (DU 730, UV/Vis Spectrophotometer, Beckman Coulter®) which had been calibrated previously by using a cell counting chamber (Hemacytometer Set, Hausser Scientific).

4.5.4 Setup and Operation of the Flow Chamber

All flow chamber experiments were conducted in a dark box (Figure 4-1) to ensure minimal interference between bacterial bioluminescence and other light sources. To avoid contamination of tubes and inlet/outlet ports, three way valves were used at each connection port to facilitate alcohol sterilization as needed. Autoclaved medium was aerated with an aquarium air pump (Whisper®, Tetra Holding, US) connected to a diffuser.

Figure 4-2 displays the custom-designed flow chamber used in our studies. It consisted of a 9.0 cm by 14.0 cm by 1.0 cm aluminum spacer with two 10 cm by 1 cm by 1 cm regions which had been removed so they could house porous medium, and was equipped with inlet and outlet ports. The aluminum spacer was sandwiched between two transparent acrylic plates sealed to the spacer by fluorocarbon rubber (Viton®, Dupont Dow Elastomers). The bottom plate was drilled with five holes in a row, located 1, 3, 5, 7, and 9 cm from the inlet (see Figure 4-2) to monitor the pressure distribution by using a calibrated high accuracy micro-machined silicone sensor pressure transducer (PX409-100GUSB, Omega). These ports were also used to inoculate the porous medium at different locations within the chamber.
Figure 4-1: Dark box used to provide completely dark environment to ensure minimal interference of bacterial bioluminescence by other light sources.
Figure 4-2: Experimental setup showing the custom designed flow chamber. The aluminum spacer was sandwiched between two transparent acrylic plates with gaskets serving to ensure a tight seal. The bottom acrylic plate was drilled with five holes in a row, located at 1, 3, 5, 7, and 9 cm from the inlet to monitor the local distribution of pressure along the chamber. The chamber was operated in a horizontal orientation. A Mariotte tube was used to house the biofilm growth medium used in the chamber. The inlet valves were used to ensure that, at any time, the medium only entered one porous medium channel, and not the other.

Each void space in the aluminum spacer was filled with spherical acid-washed glass beads ranging in diameter from 425 to 600 µm (30-40 US sieve) (Sigma-Aldrich, G8772), with particle density equal to 2.6 g/cm³. The chamber was outfitted with 0.1 mm nylon mesh screens at both the inlet and outlet of the porous medium. Also, fluid fluxes
under different pressure gradients were measured to determine the hydraulic conductivity of the packed porous medium as follows:

\[ q_w = -\frac{A K h}{L} \]  (4.1)

where \( q_w \) is volumetric flow rate of the water phase, \( A \) is cross-sectional area of the porous medium, \( K \) is hydraulic conductivity, and \( h \) is hydraulic head difference applied over a length \( L \).

A Mariotte tube (SMS®, Arizona, USA) was used to maintain a constant pressure difference between chamber inflow and outflow, thereby enabling the hydraulic conductivity of the porous medium to be determined. After inoculation and during the biofilm growth and development period, a constant hydraulic pressure head of 7 cm was applied across the porous medium resulting in an average hydraulic gradient of 0.7 m/m.

Prior to use, the chamber was autoclaved after assembly and allowed to dry and equilibrate to room temperature for 24 hours in a laminar flow biosafety cabinet (Forma Class II A2; Thermo Electron Corporation). After drying, the chamber was sealed and all autoclaved fittings and tubing connectors were installed, taking care to ensure sterility. The porous medium was then filled with sterile deionized water via a Gilson Minipuls 3 peristaltic pump while the chamber was maintained in a vertical position.

After filling, the chamber was placed horizontally in the dark box and a tracer test was conducted to ensure uniform flow across the porous medium. Movement of the tracer pulse indicated that the chamber was packed uniformly, as revealed by a flat leading edge profile, and breakthrough studies confirmed that the chamber was homogeneously packed and no preferential flow path existed. Following the tracer test, the chamber was flushed...
with biofilm growth medium and subsequently, the experiment was initiated by inoculation of 0.25 mL of cell culture inoculum (6x10^6 cells/mL) into the porous medium via the center port on the bottom plate of the chamber. This resulted in a 0.5 cm radius inoculation region around the injection port with a bulk volume of approximately 0.8 cm³. The inoculation region was visualized using Brilliant Blue FCF dye (ACROS Organics) which had been included in the inoculum. To prevent bacterial washout, continuous background flow of biofilm growth medium was not initiated until 24 hours after inoculation to provide sufficient time for bacterial attachment to the porous matrix. The fluid flow rate was monitored by using calibrated in-line flowmeters (BEL-ART Riteflow®) at both the inlet and outlet of the chamber.

4.5.5 Bioluminescence Imaging

Images of bioluminescence emitted from biofilms within the chamber were taken once every 24 hours. Prior to taking images, the medium flowing through the inlet port was switched from biofilm growth medium to induction medium for a period of 90 minutes. All inlet flow was then blocked and a bioluminescence image was taken using a 14-bit digital CCD camera (Progres MFcool CCD camera, Jenoptik, Germany) with an interline 2/3" Sony ICX285AL 1.4 megapixel progressive scan monochrome CCD sensor (encased by a hermetically sealed, nitrogen-flushed capsule cooled by a Peltier element, a heat sink and a fan). This camera was outfitted with a Computar Megapixel lens with 35 mm focal length and f/1.4 focal ratio. Images were taken with a longpass UV filter (Edmund Optics, 25.5 mm) which permitted the passage of all fluorescent light while protecting the lens.
To take images, the camera was located 40 cm above the chamber and controlled by the CapturePro software (ProgRes® CapturePro 2.7.7, Jenoptik, Germany). In all the experiments, a 5 min exposure time with full aperture (f1/4) was used. The conditions and procedures used to obtain each image were consistent for all experiments making it possible to compare different bioluminescence images on the basis of bioluminescence intensity (BI), and therefore, evaluate the impact of different parameters on cell bioluminescence. A background image was taken prior to induction and then subtracted from each image recorded after induction (using CapturePro software) to minimize the effects of surrounding optical noise.

4.5.6 Image Processing

4.5.6.1 Assignment of Bioluminescence Intensity Values

Each image was taken in grayscale, with a direct relationship between BI and pixel intensity. Seven distinct gray levels could be electronically detected in the bioluminescence images. Thus, each pixel in a grayscale image was assigned an integer value from 0 (no bioluminescence detected) to 6 (maximum bioluminescence detected). This assignment allowed each 2D image to be treated as a 2D matrix of gray level values based on the scale used by the camera. Each 2D matrix was then analyzed by using MATLAB® Image Processing Toolbox Version 7.8 (MATLAB® R2009a, MathWorks Inc.) (Appendix J). However, the differences in darkness between pixels in the raw images were difficult to discern with the naked eye. To improve visual quality, we decided to take advantage of the 256 gray levels (8 bit grayscale color map) in the MATLAB® software package. The camera-based integer values in each 2D matrix were
Figure 4-3: The porous medium flow chamber was divided into four zones as shown at the top of this figure. The flow inlet was located to the left of Zone 1, and the outlet was to the right of Zone 4. The chamber was inoculated on day zero at the interface between Zones 2 and 3 (marked by the circle). Bioluminescence images were obtained daily by introducing induction medium through the inlet, causing the cells in the biofilm to fluoresce. The bioluminescence images shown here represent the evolving biofilm growth pattern over the course of 8 days. Scale indicates increasing bioluminescence intensity from 0 to 6.

scaled to the entire MATLAB\textsuperscript{®} grayscale color map by a common practice in image processing known as gray contrast enhancement with a linear contrast stretch (Schowengerdt, 1983; Al-amri et al., 2010). Thus, the original gray scale was expanded to the scale shown in Figure 4-3 where a value of 0 again refers to no bioluminescence and a value of 6 refers to maximum bioluminescence (please see Appendix J for more
details). Rescaling the gray levels allowed for small differences in gray level intensities
detected by the camera to be visually discerned by the naked eye in images produced by
the image processing software.

4.5.6.2 Image Segmentation

To analyze bioluminescence images and verify whether the detected BI could be related
to hydraulic conductivity, the average BI in each zone was required. However, to have
statistically meaningful outcomes from a series of images, a consistent method should be
utilized to determine these average values. Based on results reported by Sezgin and
Sankur (2004), the Otsu approach (Otsu, 1979) was selected to obtain optimum threshold
values (i.e. average BI in each bioluminescence image). Thresholding is an image
segmentation method that can be used to separate an image into distinct components
(Yang et al., 2001; Sezgin and Sankur, 2004). In this technique, target elements are
isolated from background regions based on gray level distribution amongst image
elements. The method separates data by grouping all pixels with intensities greater than a
certain gray level into objects and all other pixels into background (Otsu, 1979). In this
technique, the procedure iterates over all possible thresholds to determine an optimal
value based on minimization of within-class variances (Sezgin and Sankur, 2004) as
follows (please see Appendix J for MATLAB® code):

1. An intensity level is selected as the threshold value ($\tau$)

2. The bioluminescence image is segmented into object (O) and background (B) classes,
and then the average BI for each zone in the porous medium chamber (see Figure 4-3
for the locations of the zones) is computed. The average object bioluminescence intensity ($BI_O$) is calculated as:

$$BI_O = \frac{\sum_{n=1}^{256} (n^{th} BI)(\text{Frequency of } n^{th} BI)}{\sum_{n=1}^{256} (\text{Frequency of } n^{th} BI)}$$

(4.2)

The average background bioluminescence intensity ($BI_B$) is found from:

$$BI_B = \frac{\sum_{n=1}^{\tau} (n^{th} BI)(\text{Frequency of } n^{th} BI)}{\sum_{n=1}^{\tau} (\text{Frequency of } n^{th} BI)}$$

(4.3)

3. A within-class variance, defined as a weighted summation of variances, is computed:

$$\delta_i^2(\Theta) = \omega_i(\tau)\delta_O^2(\Theta) + \omega_B(\tau)\delta_B^2(\Theta)$$

(4.4)

where $\omega_i(\Theta)$ and $\delta_i^2(\Theta)$ represent probability and variance of object ($i = O$) and background ($i = B$) segments versus the threshold value, $\Theta_i$, respectively.
4. The optimal threshold value was then selected as the BI most closely corresponding to the minimum within-class variance.

Based on this procedure, the selected threshold value was considered to be the overall BI for a particular zone in the porous medium. Threshold values were used to convert bioluminescence images to binary images, which identified that portion of the pore space filled with biofilm, thereby showing the distribution of biofilm within the porous medium (please see Appendix J for MATLAB® code).

4.5.7 Additional Measurements

To investigate the characteristics of biofilm throughout the chamber at the end of an experiment, seven 0.15 cm$^3$ samples of porous medium were collected from each zone using a 4.5 mm diameter hollow tube. Each porous medium sample was placed into a separate test tube containing a known volume of PBS and vortexed and sonicated repeatedly to detach biofilm from glass beads (Heersink, 2003; Trachoo, 2004) (please see Appendix C for more details). Detachment was verified following this procedure by viewing the beads microscopically to ensure that they were clean and not covered in biofilm (Trachoo, 2004; Johnson et al., 2010). The resulting biofilm samples, as well as the chamber effluent samples collected at the outlet via a three way valve (Figure 4-2), were analyzed for bacterial cell density, total protein, and dissolved oxygen levels.

4.5.7.1 Dissolved Oxygen Determination

A dissolved oxygen test kit (CHEMets®, CHEMetrics Inc.) based on the Indigo Carmine assay (Loomis, 1954) was used to determine the amount of dissolved oxygen in the chamber effluent. In this method, the dissolved oxygen oxidizes the leuco-base of indigo
carmine to produce a blue-green dye, the intensity of which can be measured spectrophotometrically. Here, measured absorbance values (wavelength 470 nm) were compared against standard solutions ranging from 0 to 10 mg/L oxygen (provided by CHEMets®, CHEMetrics Inc.).

4.5.7.2 Quantification of Bacterial Cell Populations

The bacterial population density in each porous medium sample was determined by measuring the mass of total DNA based on the selective binding of DNA to a silica-based membrane. Proteinase K and chaotropic salt were used to lyse the cells and degrade protein, allowing DNA in the chaotropic salt to be easily bound by the silica-based membrane (Qubit® dsDNA Assay Kit, Invitrogen, Oregon, USA). In addition, RNase A was added to the lysate to degrade RNA and minimize RNA contamination of the purified DNA samples (please see Appendix F for more details).

To correlate the total DNA collected in a sample to the number of cells present in that sample, it was necessary to first calculate the average DNA content per cell. By summing the mass of the PUTK21 plasmid used in HK44 and the published mass of DNA in wild type *Pseudomonas fluorescens*, the total DNA content per cell was calculated to be equal to 7.843x10^{-15} g (King et al., 1990). A correlation between mass of DNA and corresponding number of bacterial cells was generated by manual cell counting (at least 360 bacteria per slide were counted in triplicate for statistical relevance) using a counting chamber (Hemacytometer Set, Hausser Scientific) (Appendix G).
4.5.7.3 Protein Measurement

Protein can be found both within cells and EPS. The total protein concentration in porous medium samples was determined by using the bicinchoninic acid (BCA) method (Smith et al., 1985). Bacterial protein extraction lysis buffer (Bacterial–PELB, GBiosiences®) was used to extract intracellular proteins. Absorbance of light (wavelength 562 nm) against the fresh reagent was measured by the same spectrophotometer described above and compared to a standard curve generated by using bovine serum albumin (BSA) (please see Appendix H for more details). Also, to differentiate between EPS and intracellular proteins, a liquid batch culture of HK44 was grown in stock culture growth medium on a rotary shaker (Heidolph Unimax 2010, Germany) at 150 rpm and room temperature. Three samples (5 mL each) were collected at different growth stages and bacteria were harvested from each sample by centrifugation (5000 rcf, 4°C for 20 minutes) and washed twice with PBS. Total intracellular protein was then measured by using the same method described above. In addition, protein content of individual cells, obtained from the biofilm samples, was evaluated to identify differences in protein content between planktonic and biofilm bacteria.

4.5.7.4 Direct Visualization

Biofilm samples collected from each zone were visualized by phase-contrast microscopy (Axio Observer.A1, Zeiss, Germany) to investigate the effect of environmental conditions on the biofilm structure.
4.6 Results and Discussions

4.6.1 Spatiotemporal Development of Biofilm in Porous Medium

Figure 4-3 shows bioluminescence images obtained for the evolving biofilm under flow in a porous medium over a period of 8 days. This experiment, with identical conditions, was repeated five times with very similar results (see Figures I-1 and I-2 in Appendix I for data from other trials). The bioluminescent response of the cells was recorded after injection of induction medium from the left side through the chamber inlet (i.e. one-way induction). The first image (Day 1) of the chamber was taken 24 hours after being inoculated, but prior to injection of growth medium. This image was used to visualize the region that was initially inoculated (marked "inoculation region" in Figure 4-3). Our batch experiments showed that maximum HK44 bioluminescence occurred after approximately 90 minutes of induction and remained constant for at least 30 minutes in the oxygen-saturated induction medium. Thus, in future experiments, injection of the induction medium was maintained for 90 minutes.

The bioluminescence images revealed that, during the first 3 days, biofilm growth was confined to the inoculation region, and spatial spread of biofilm throughout the chamber was negligible. However, on Day 4, once biofilm had grown sufficiently within the inoculation region, it extended almost symmetrically into Zones 2 and 3. From the Day 4 image in Figure 4-3, small independent areas of bioluminescence downstream of the inoculation zone are evident. Biofilm distribution in the downstream sections (Zones 3 and 4) can be explained by convective transport of detached biomass (detached cells and biofilm clusters) from upstream zones which reattach in downstream regions. The discontinuous and non-homogeneous growth pattern of biofilm in downstream sections
could have resulted from random localization of detached cells and non-homogeneous flow (i.e. nutrient availability) due to biofilm growth and hydraulic conductivity changes in upstream zones.

After Day 4, biofilm continued to grow and spread throughout the chamber, with BI indicating that biofilm saturation (fraction of pore space occupied by biofilm) was highest upstream of the inoculation site towards the nutrient inlet port. Similar growth patterns have been reported in other biofilm studies indicating that biofilm tends to migrate primarily from initially inoculated regions toward inlet ports (deLeo and Baveye, 1997; Kildsgaard and Engesgaard, 2002; Seifter and Engesgaard, 2012). Migration of cells against a pressure gradient cannot be explained simply by convective transport, and therefore, dynamic interactions between biofilm and fluid flow must be taken into account. One possible explanation for this observation is that as the flowing liquid approaches the biofilm mass, it decelerates and forms a stagnation point at the nose of the mass, thereby creating slow flow conditions just upstream of the biomass. This creates an active growth zone immediately upstream of the biofilm mass due to lower flowrates and the presence of fresh growth medium which promotes biofilm growth towards the inlet. Also, bacterial movement, and consequently biofilm growth pattern, may be influenced by bacterial chemotaxis in response to nutrient concentration gradients.

Another important feature of the images was emergence of dark regions on Days 6 and 8 in sections previously shown to be occupied by biofilm on Day 5. Quantification of cells obtained by destructive sampling at the end of the experiment (Figure 4-4; see also Figure I-1 in Appendix I) indicated relatively high cell population densities within regions of
Zones 3 and 4 even though these regions appeared dark in the images on Day 8 (Figure 4-3) and had earlier emitted detectable bioluminescence. For example, the region peripheral to the inoculation port changed from being highly luminescent on Day 5 to less so on Day 6 (Figure 4-3). Low flowrates and moderately mature biofilm in these sections suggested that the reduction in detected BI was not caused by cell loss. Rather, as biofilm saturation increased, penetration of induction medium into these sections was reduced and redirected around these low permeability regions, thereby not reaching the cells in sufficient quantities needed for them to bioluminesce. Thus, unlike those microbes that contain constitutively expressed fluorescent proteins, bioluminescence intensity variations in microbial populations containing inducible lux genes can be used to represent varying degrees of biofouling in biofilm containing regions. Biofouling is typically diagnosed indirectly in experimental investigations or biological processes by the impact that it has on hydraulic conductivity or product quality/quantity (Flemming et al., 2011).
To verify that cells within dark regions that were previously luminescent retained their ability to emit bioluminescence, they were sampled at the conclusion of the flow experiment and cultivated in fresh stock culture growth medium. Bioluminescence activities recorded after exposure to induction medium confirmed the presence of active lux-genes in the culture obtained using cells from dark regions (Figure 4-3 – Zones 3 and 4). This suggests that the lack of bioluminescence in the flow chamber was not due to a change within the cells, but rather the absence of one or more factors that induce bioluminescence. Within biofilm-saturated zones, mass transfer is dominated by diffusion and dispersion. Consequently, fluids do not readily penetrate into biofilm-saturated regions due to their lower permeability. Thus, during HK44 induction, it is likely that induction medium bypassed these bioclogged regions, instead penetrating regions with higher permeabilities. The relative lack of induction agent within biofilm-saturated regions negatively impacts the ability to visualize cells in these regions by using the described induction method.

Metabolism related to bioluminescence requires high levels of molecular oxygen (Uesungi et al., 2001; Oates et al., 2005). During the induction phase on Day 8, dissolved oxygen measurements at the chamber outlet revealed a reduction to less than 3 mg/L from its inlet concentration of approximately 9 mg/L. This relatively large reduction in oxygen concentration is explained by the high oxygen consumption required for bioluminescence. Dissolved oxygen in the chamber effluent was detected as high as 7-8 mg/L prior to switching the background flow to induction medium which indicates that HK44 bioluminescence depends upon oxygen availability. Thus, lack of bioluminescence in regions with high biofilm saturation may have been due, in part, to low oxygen...
concentration in addition to lack of induction agent, consistent with other studies on the bioluminescent response of lux-genes (Oates et al., 2005).

4.6.2 Two-Way Induction

To reduce the negative effects of oxygen depletion and lack of inducer availability on bioluminescence, especially downstream of bioclogged regions, another set of experiments was conducted in which images were first taken as described above, and then again with the flow reversed; that is, after being fed through the inlet and flowing towards the outlet as shown in Figure 4-2, the induction medium was fed through the outlet and allowed to flow back through the chamber towards the inlet. The method of injecting fluids into the chamber was identical for both one-way (described in the Materials and Methods - Section 4.5.4) and two-way induction. This was done to overcome downstream oxygen limitations that would occur if the medium was only fed through the inlet, which in turn would enable improved imaging of cells in Zones 3 and 4. These results are summarized in Figure 4-5. According to the results obtained from the inlet side induction (Figure 4-5A), practically the same bioluminescence behaviors as recorded in the previously described experiment (Figure 4-3) were observed. However, injection of induction medium from the outlet side (Figure 4-5B) revealed a different bioluminescence pattern throughout the chamber. Similar results were obtained in a duplicate experiment (see Figure I-3 in Appendix I).

Compared to injection of induction medium from the inlet side, Zones 1 and 2 now exhibited lower BIs, whereas Zones 3 and 4 exhibited much higher BIs. Again, these results were obtained because bioclogging affected the flow of induction agent in Zones 1
Figure 4-5: Bioluminescence response of the biofilm in the porous medium over the course of 8 days by (A) induction from the left (inlet), and (B) induction from the right (outlet) side of the flow chamber. The chamber was initially inoculated at the interface between Zones 2 and 3 (marked by the circle). Scale indicates increasing bioluminescence intensity from 0 to 6.
and 2. Even when induction medium was able to penetrate areas in these two zones, oxygen would have been relatively low due to consumption by cells in Zones 3 and 4. In contrast, feeding induction medium through the outlet resulted in relatively high concentrations of induction agent and oxygen in Zones 3 and 4.

To obtain a better understanding of overall biofilm growth and development throughout the porous medium, images obtained from inlet and outlet side inductions were merged together. A merged image was constructed by comparing the BI of a given pixel in the inlet side induction image to the BI of the corresponding pixel in the outlet side induction image. The higher of the two intensities was used to generate the final merged image (Figure 4-6A; see also Figure I3 in Appendix I). Obtained merged image was a more accurate representation of biofilm growth in the chamber than either of the two images alone from which it was constructed.

Figure 4-6: (A) Overall bioluminescence image on Day 8 obtained by merging images captured following both inlet and outlet induction. Scale indicates increasing bioluminescence intensity from 0 to 6. (B) Binary image obtained by applying the Otsu approach to determine a threshold value.
4.6.3 Analysis of Bioluminescence Images

To correlate the measured BI of a merged image to biofilm characteristics and hydraulic conductivity, it was necessary to develop methods to accurately estimate the overall amount of biofilm within each zone of the chamber. To accomplish this, bioluminescence images were first subjected to an image segmentation process to distinguish biofilm from void space that did not contain biofilm. Figure 4-6B shows the binary image obtained by segmenting the final merged bioluminescence images by using a threshold value determined using the Otsu approach. Also, threshold values, representing the average BI in the corresponding region, were calculated for each zone to show average BI variations throughout the 8 day experiment (Figure 4-7). Note that the threshold values were normalized by the maximum gray level because MATLAB® does not accept threshold values larger than unity.

Figure 4-7: Calculated average bioluminescence intensity for each zone during the course of 8 days experiment.
4.6.4 Biological Analyses

From a qualitative analysis of the bioluminescence images, it appeared that bacteria tended to grow and form biofilm preferentially from the inoculation site towards the inlet rather than downstream into Zones 3 and 4. To determine if the images could be used to quantitatively assess the properties of the biofilm, assays were carried out in an attempt to correlate BIs to cell numbers and protein concentrations within the chamber. The results of these assays, summarized in Figures 4-4 and 4-8, respectively, show that in upstream Zones 1 and 2, with higher BI, the average numbers of cells were higher than those in the downstream zones. Also, microscopic investigation of biofilm samples revealed a denser bacterial population upstream of the inoculation site compared to downstream sections (Figure 4-9).

![Figure 4-8: Protein concentrations in seven samples collected at the end of the experiment for each zone.](image)
The measured cell numbers were plotted against calculated BIs for each zone, as shown in Figure 4-10. A power function was determined to be the most accurate expression ($R^2 = 0.97$) to describe the relationship between these two variables. Based upon Figure 4-10 (see also Figure I-3 in Appendix I for more data), the scaling exponent was calculated as 3.11, meaning that biomass accumulation affected microbial bioluminescence activity in the porous medium.

West and Brown (2005) conducted a theoretical investigation to explain allometric scaling relationships between biological activities and biomass accumulation in biological systems. They hypothesized that in any biological system, microbial populations could be sustained by transport of materials through linear networks that branch to supply metabolic substrates and remove waste products from all parts of a biomass. Accordingly, during the initial stages of biomass accumulation, it would be expected that the metabolic activity of microorganisms (such as emission of bioluminescence) would increase isometrically with an increase in the number of cells.
Figure 4-10: Number of cells versus average bioluminescence intensity calculated on day 8 showing a power function relationship between cell numbers and average bioluminescence intensities at each zone.

However, evolution of the biomass over time would put geometric constraints on the accessibility of network branches, thereby restricting substrate (including oxygen and induction factor) distribution, which in turn would cause the relationship between metabolic activity and cell number to shift from being linear to sub-linear. West and Brown (2005) showed that for most biological activities, the allometric scaling exponent would be in the range of 1.3 to 4, which was consistent with the scaling exponent of 3.11 found in the present study for microbial bioluminescence activity. In addition, a second possible contributor to the observed nonlinearity could be that at higher cell numbers, the emitted bioluminescence from cells within a biomass could be increasingly masked by the cells and EPS that make up the outer layers of the biomass.

Protein concentration measurements from the four zones, did not correlate directly to cell numbers, with the ratio of protein mass to cell numbers higher in Zones 3 and 4.
compared to Zones 1 and 2 (Figure 4-8). In early biofilm studies, EPS was believed to be composed mostly of polysaccharides. However, more recent investigations suggest that protein is also present in EPS in considerable amounts. For example, Nielsen et al. (1997) quantified the EPS fraction of biofilms in trickling filters and biofilters and concluded that protein is abundant in biofilm EPS. Other investigations on the EPS of active sludge biofilms also revealed that protein is present in large quantities (Dignac et al., 1998; Zhang and Bishop, 2003; Metzger et al., 2009; Flemming and Wingender, 2010). The high protein-to-cell ratio measured in Zones 3 and 4, therefore, could be explained by the fact that in our studies, we had measured total protein (i.e. intracellular and EPS protein) and not just intracellular protein. Since cell densities in Zones 3 and 4 were lower than in Zones 1 and 2, but total proteins levels were much greater, it suggests that conditions in downstream regions promoted greater EPS secretion and less cellular proliferation than the prevalent environmental conditions in upstream regions.

Given that we measured the number of cells within biofilm samples, and found the average intracellular protein content to be $1.12 \times 10^{-13}$ g of protein per cell (regardless of whether the cells were suspended in a planktonic manner or within a biofilm), the proportion of total protein that was actually extracellular (i.e. from the EPS) in each sample could be calculated. These results are summarized in Figure 4-8. It has been shown that under adverse environmental conditions, bacteria consume available nutrients to secrete EPS rather than proliferate in order to provide mechanical stability to their surroundings, thereby enabling better protection against environmental stresses (Flemming and Wingender, 2010). In addition, biofilm EPS acts as an extracellular nutrient store which can later be biodegraded by bacterial cells when needed (Zhang and
Bishop, 2003). In our studies, the high ratio of EPS to cell count in downstream regions is explained by unfavourable environmental conditions with respect to nutrient and oxygen availability. High biofilm content in upstream regions significantly interfere with penetration of biofilm growth medium flow to downstream regions, thereby creating stressful conditions known to promote EPS production.

4.6.5 Porous Medium Hydraulic Properties

Based on mass measurements and density of solid particles, the total porosity of the medium was calculated to be 38% at the beginning of the experiment. Also, based on flux measurements at different pressure head values, the hydraulic conductivity of the system was estimated at $6.05 \times 10^{-5}$ m/s (Equation 4.1). Over 8 days, under constant pressure head, the measured water flow rate through the chamber decreased gradually from 0.248 mL/min at the beginning of the experiment to 0.110 mL/min by the end (Figure 4-11), indicating a reduction in hydraulic conductivity.

As biofilm develops and occupies a greater proportion of pore space, the remaining pore space available for fluid flow decreases. Consequently, the reduction in hydraulic conductivity at constant pressure head leads to a drop in the fluid flux through the porous medium. Figure 4-12 (see also Figure I-3 in Appendix I) shows that hydraulic conductivity, represented by fluid flow rates, were correlated to the overall measured BI in an image, calculated as threshold values by the Otsu approach, over the entire porous medium. Based upon the allometric scaling relationship between BI and number of cells (Figure 4-10), the obtained linear correlation between BI and flow rate (Figure 4-12) implies a nonlinear relationship between hydraulic conductivity and the number of cells in porous media.
Figure 4-11: During the course of 8 days experiment, fluid flow rate through the porous medium, decreased gradually from 0.248 mL/min at the beginning to 0.110 mL/min by the end.

Figure 4-12: Flow rates through the porous medium versus overall bioluminescence intensities calculated for the entire porous medium showing a linear correlation.
One possibility for this nonlinear relationship can be diffusion of nutrients to the biofilm saturated zones which sustains microbial growth in such regions. However, higher biomass accumulation in these clogged regions will have trivial effects on overall hydraulic conductivity, thereby resulting in a nonlinear relationship between these two parameters.

To confirm the relationship between BI and hydraulic conductivity, after each bioluminescence image was recorded, the accompanying hydraulic head was recorded along the porous medium via the pressure ports at the bottom of the flow chamber (Figure 4-13). Based on the hydraulic head profiles, the highest pressure drop observed after 6 days was in the vicinity of the initially inoculated region, as revealed by a sharp pressure gradient across Zones 2 and 3 (3 to 7 cm from the inlet). The overall hydraulic head reduction observed throughout the porous medium on Day 8 could be explained by clogging of the inlet screen.

Figure 4-13: Hydraulic head profiles measured along the porous media via pressure ports at the bottom of the flow chamber at 1, 3, 5, 7, and 9 cm from the inlet.
In addition, based on pressure gradients across each zone and measured fluid flow rates, Equation 4.1 was used to calculate hydraulic conductivity changes and these values were plotted against the time dependent variations in BI in each zone (Figure 4-14). The correlation revealed between hydraulic conductivity and average BI indicated that the overall BI could be used to evaluate hydraulic conductivity in each zone. Such a correlation was not unexpected as the development of biofilm restricts fluid flow and mass transfer, in turn causing a drop in BI.

Figure 4-14: Hydraulic conductivity variations during the course of 8 days experiment combined with average bioluminescence intensities calculated for each zone showing possible correlations between average BI and porous medium hydraulic properties.
4.7 Conclusions

We have shown that it is possible to simultaneously evaluate biofilm development and its corresponding effects on porous media hydraulic conductivity by using real time imaging in conjunction with a model bioluminescent organism. Microbial bioluminescence intensities, calculated through image analysis, were correlated to the number of cells and hydraulic conductivity changes in corresponding regions. Such correlations can be used to determine important characteristics in porous medium based biofilm systems (such as cell density, biofilm saturation, and hydraulic conductivity) in real-time, and provide an opportunity to better understand, predict and control interactions between biofilm growth and hydraulic properties, thereby facilitating the development of porous medium based biofilm applications.
5.1 Preface

The present thesis is based on refereed papers that have already been published or are in the process of publication. Unavoidably, there is some repetition between the chapters, particularly in the Introduction to each chapter and experimental procedures. Therefore, in this chapter, sections of *Introduction* and *Materials and Methods* may be safely skipped without loss of coherence. In addition, more comprehensive details of experimental procedures used to prepare culture media, sample the porous media, prepare biofilm samples, and determine bacterial population, as well as the assays used to measure bacterial DNA and protein concentration are provided in Appendices A through G. Also, MATLAB codes used to process the bioluminescence images are summarized in Appendix J.)
5.2 Abstract

Biofilm formation in natural and engineered systems can significantly impact hydrodynamics in porous structures by affecting porosity, permeability, and mass transfer of solutes. To better understand and characterize how biofilms influence hydrodynamic properties in porous medium, *Pseudomonas fluorescens* HK44, a genetically engineered bioluminescent bacterial strain was used to quantify microbial population characteristics and biofilm properties in a translucent porous medium. Using imaging technology, bacterial bioluminescence was detected and related to bacterial cell density, hydraulic conductivity, and fraction of void space occupied by biofilm (i.e. biofilm saturation). The results revealed that under different growth conditions, power functions with similar allometric scaling exponents can be used to correlate bioluminescence intensity to bacterial cell density. This work also resulted in the construction of relative hydraulic conductivity curves which will facilitate the development of new models to describe the dynamic behavior of biofilm and fluid flow in porous media.
5.3 Introduction

Biofilms are structured communities of microorganisms embedded in a self-produced organic matrix of extracellular polymeric substances (EPS). In porous structures, which inherently have high surface area to volume ratios in comparison to non-porous structures, microbes can quickly colonize pore surfaces and start to form biofilms rather than remaining in a planktonic state (van Loosdrecht et al., 1990; Bouwer et al., 2000). With sufficient nutrient supply and metabolic waste removal, biofilms can progressively accumulate within a pore space (Taylor and Jaffe, 1990a; Cunningham et al., 1991; Baveye et al., 1998; Bozorg et al., 2011), making it increasingly difficult for fluids to flow through the porous structure. In other words, biofilm growth in porous media leads to bioclogging that reduces overall hydraulic conductivity.

Biofilm growth has been shown to have an impact on porous medium permeability reduction, fluid transport, mass transfer, and microbial activity (Seki et al., 1998; Bielefeldt et al., 2002; Kildsgaard and Engesgaard, 2002; Thullner et al., 2002; Arnon et al., 2005; Paelic et al., 2007; Seifert and Engesgaard, 2007; Bozorg et al., 2012). In general, these studies have revealed that the rate of biofilm growth and spread in a porous medium is tied to geohydrological characteristics (e.g. the permeability and porosity distributions) of the porous medium itself. Biofilm formation and fluid flow within a porous medium can be considered to be interdependent. The development of biofilm can impact key geohydrological characteristics of a pore network, such as effective porosity (the fraction of the pore space available to contribute to fluid flow) and permeability, thereby affecting fluid flow. Since these fluids carry dissolved components that affect cell survival and proliferation, such as nutrients and metabolic wastes, the change in fluid
flow patterns can, in turn, significantly influence the spatiotemporal development of biofilm.

Biofilms are often considered to be detrimental and undesirable as they can negatively impact industrial processes that rely on the flow of fluids in porous media. However, the active management of biofilm growth in porous media could provide significant opportunities to improve the performance of those industrial and environmental processes that use biofilms to achieve a process goal, such as in situ bioremediation, reactive biobarriers, wastewater treatment, enhanced oil recovery, and carbon sequestration (Shaw et al., 1985; Harvey et al., 1989; Thomas and Ward, 1989; Madsen 1991; Lappin-Scott et al., 1998; Nicoletta et al., 2000; Kim et al., 2006; Singh et al., 2006; Bishop, 2007; Mitchell et al., 2009). What are needed are methods that minimize the detrimental effects of biofilms in porous media while maximizing the potential benefits. Development of robust methods to engineer biofilms in porous structures requires comprehensive knowledge of the processes that affect their spatiotemporal development under different flow conditions. However, due to an inadequate understanding of the interactions between geohydrological properties, biofilm growth kinetics, multiphase flow effects, spatial variations of cell nutrients, and the impact of medium heterogeneity, even the best models are not able to accurately predict observed biofilm behavior and geohydrological changes in porous media, and thus, field scale applications of biofilm based processes are still unpredictable (Bielefeldt et al., 2002; Seifer and Engesgaard, 2007; Geesy and Mitchell, 2008; Gerlach and Cunningham, 2010).
Different models have been used to simulate biofilm growth in porous media (Baveye and Valocchi, 1989; Taylor and Jaffe, 1990b; Tan et al., 1994; Vandevivere et al., 1995; Clement et al., 1996; Noguera et al., 1999; Dupin et al., 2001; Thullner et al., 2004; Brovelli et al., 2009). The general approach used in these models has been to incorporate relationships that link porosity to hydraulic conductivity (Dupin et al., 2001; Thullner et al., 2004; Kim and Whittle, 2006) by treating biofilm as an emerging solid phase capable of changing the intrinsic porosity and permeability of a medium. Whereas such models are able to qualitatively reproduce experimental results, they are limited due to the lack of reliable correlations between porosity and permeability (Molz et al., 1986; Baveye and Valocchi, 1989; Clement et al., 1996; Baveye et al., 1998). Recently, Bozorg and colleagues (2011) introduced a new macroscopic approach to model biofilm spatiotemporal development in porous media by treating biofilm as a high viscosity liquid phase that shares pore space with a low viscosity aqueous phase. In that study, efforts were made to quantify effective conductivities of the biofilm and water phases via relative permeability curves based on biofilm saturation (fracture of pore space occupied by biofilm). However, calibration of the parameters used in this approach is challenging as it requires simultaneous evaluation of biofilm evolution and hydraulic conductivity.

Real-time quantification of biofilm characteristics and porous media geohydrological properties have been a subject of interest in porous media based processes for many years (Oostrom et al., 1998; Niemet and Selker, 2001; Seymour et al., 2004; Davit et al., 2011; Bozorg et al., 2012). A variety of approaches, such as microscopic visualization (Dupin and McCarty, 2000) and scanning electron microscopy or transmission electron microscopy (Rinck-Pfeiffer et al., 2000; Hand et al., 2008) have been used to determine
the presence, distribution, and structure of biofilm at the pore scale. Also, imaging via high resolution photography, X-ray tomography (Davit et al., 2011), nuclear magnetic resonance spectroscopy (Hoskins et al., 1999; Seymour et al., 2004), and ultrasound-based imaging techniques (Shemesh et al., 2007) have been used to non-destructively visualize biofilm growth and distribution in porous structures. Nevertheless, such technologies usually suffer from low resolution, inability to differentiate components, and limited depth penetration. Furthermore, many of these methods expose biofilms to high energy levels which can be detrimental to the microbes (Vafai, 2011).

In recent years, new approaches have been developed that capitalize on the bioluminescence emitted by certain natural and engineered microorganisms to monitor microbial processes in natural and engineered environments (Burlage et al., 1990; Shaw et al., 1992; Sanseverino et al., 1993; Flemming et al., 1994; Ripp et al., 2000; Uesugi et al., 2001; Sharp et al., 2005; Trögl et al., 2007). For instance, Sharp and coworkers (2005) used naturally luminescent bacteria in a flat-plate flow chamber to study biofilm growth under flow; whereas bacterial bioluminescence was used to track biofilm development, no quantification was made of the detected bioluminescence intensity. In another experimental study, Bozorg and colleagues (2012) used CCD camera technology to monitor growth of biofilm forming bioluminescent bacteria in a translucent porous medium. They also demonstrated the potential of inducible bacterial bioluminescence for the nondestructive evaluation of cell density and porous medium hydraulic properties in porous media.
The objective of the research being documented here was to quantify, in real-time, porous medium hydraulic conductivity and biofilm saturation, and then to use these parameters to develop relative hydraulic conductivity curves for the flowing aqueous phase.

5.4 Materials and Methods

The bioluminescent strain, porous medium, imaging system, and fluid application system have been described in detail in Chapter 4, and so will only be described here briefly.

5.4.1 Bacterial strain

The bacterial strain used in this study was the bioluminescent reporter strain *Pseudomonas fluorescens* HK44 (hereafter referred to as HK44) obtained from the University of Tennessee Center for Environmental Technology (University of Tennessee, Knoxville, TN). This strain was genetically modified previously by transposon insertion of the salicylate-inducible *luxCDABE* gene cassette and a tetracycline resistance marker, and thus, luminesces when naphthalene, salycylate, 4-methyl salycylate, or other aromatic hydrocarbons are present (King et al., 1990).

5.4.2 Culture Media

HK44 was grown for 18 h on an orbital shaker (Heidolph Unimax 2010, Germany) at 150 rpm and 25oC in an oxygen-saturated, nitrate-free growth medium consisting of MgSO4, 0.4 g/L; CaCl2.2H2O, 0.1 g/L; NH4Cl, 0.4 g/L, NaCl, 8 g/L; KCl, 0.2 g/L; NaH2PO4, 1.15 g/L; K2HPO4, 0.26 g/L; HCl, 0.00366 g/L; FeSO4.7H2O, 0.021 g/L; H3BO3, 0.0003 g/L; MnCl2.4H2O, 0.001 g/L; CoCl2.6H2O, 0.0019 g/L; NiCl2.6H2O, 0.00024 g/L;
CuCl$_2$.2H$_2$O, 0.00002 g/L; Na$_2$EDTA.2H$_2$O, 0.01 g/L; ZnSO$_4$.7H$_2$O, 0.00144 g/L; Na$_2$MoO$_4$.2H$_2$O, 0.00036 g/L. Glucose was added to a final concentration of 1.0 g/L as the main carbon source. Stock solutions were prepared and autoclaved at 121°C for 20 minutes, and then stored at 4°C for a maximum of 2 weeks (please see Appendix A for more details). The pH of stock solutions was adjusted to 7.20 ± 0.05 using 1 M NaOH. Also, as HK44 contains a tetracycline resistance marker, all media used in this study were supplemented with 30 mg/L tetracycline to ensure plasmid maintenance. Tetracycline solution (EMD Chemicals, OmniPur® EM-8990) was prepared in 50% ethanol (v/v with water) solution and added via a 0.2 μm filter to autoclaved media. Also, to induce luminescence, an induction medium was prepared by removing all phosphate sources from the growth medium, and adding 0.1 g/L (final concentration) of salicylate. The removal of phosphorous restricted HK44 proliferation without compromising the ability of these cells to survive and metabolize (Webb et al., 1997; Oates et al., 2005), and the presence of salicylate caused the cells to bioluminesce by inducing the lux genes.

5.4.3 Porous Media

In this study, translucent acid-washed glass beads with a particle density equal to 2.597 g/cm$^3$ (Sigma–Aldrich), and ranging in diameter between 425 and 600 μm (Sigma–Aldrich, G8772) were used. The porosity of the bead pack was determined using the equation (Fetter, 1994):

$$\varphi = 1 - \frac{\rho_{bulk}}{\rho_{particle}}$$

(5.1)
where $\rho_{\text{particle}}$ and $\rho_{\text{bulk}}$ are particle and measured bulk density of the glass beads, and $\varphi$ is the porosity of porous medium. Prior to use, the glass beads were washed twice with distilled water to remove fines and autoclaved at 121°C for 20 minutes.

### 5.4.4 Dissolved Oxygen Measurement

An oxygen microelectrode (MI-730, Microelectrodes Inc.) was used to monitor oxygen concentration during periods of bacterial growth and bioluminescence in batch and continuously fed cultures. Also, in the flow chamber, an inline microelectrode was placed at the chamber outlet for real-time evaluation of oxygen concentration during biofilm growth and bacterial bioluminescence under flow conditions. The electrode was connected via an amplifier to an eDAQ Data Acquisition System (eDAQ PTY LTD, Australia) interfaced to a computer through a USB-port. Two separate media with 0% and 100% oxygen saturation respectively were used to calibrate the electrode. Autoclaved media were aerated with an aquarium air pump (Whisper® Tetra Holding, US) connected to a diffuser to supply sufficient oxygen for growth and bioluminescence of HK44.

### 5.4.5 Imaging

A 14-bit digital charged-coupled device (CCD) camera (Progres MFcool, Jenoptik, Germany) with interline 2/3" Sony ICX285AL 1.4 megapixel progressive scan monochrome CCD sensor (encased by a hermetically sealed, nitrogen-flushed capsule, cooled by a Peltier element, a heat sink and a fan) equipped with a Computar Megapixel lens with 35 mm focal length and f/1.4 focal ratio was used to capture grayscale bioluminescence images. The CCD camera was positioned 40 cm above the porous
media. All experiments were conducted in a dark box. CapturePro software (ProgRes® CapturePro 2.7.7, Jenoptik, Germany) was used to control and program the CCD camera. Throughout the experiments, all images were acquired with a 5 minute exposure time with full aperture open. The imaging process and evaluation of bioluminescence intensity (Appendix J) were previously validated and are described in detail in Chapter 4.

5.4.6 Experimental Procedures

5.4.6.1 Effect of Oxygen on Bacterial Bioluminescence

To evaluate the effect of oxygen concentration on HK44 bioluminescence activity, liquid batch cultures of HK44 were grown on a rotary shaker (Heidolph Unimax 2010, Germany) at 150 rpm and room temperature. After 18 hours, bacteria were harvested by centrifugation at 5000 relative centrifugal force (rcf) for 20 minutes (Beckman Coulter®, X-22R) and washed twice with phosphate buffered saline (PBS). Known cell densities of the harvested cells were redistributed in batch culture of induction medium and subsequently, oxygen concentration and bioluminescence intensity of induced cells were determined at regular intervals. All cell densities were evaluated by measuring light absorbance at 550 nm (Appendix E) in a spectrophotometer (DU 730, UV/Vis Spectrophotometer, Beckman Coulter®) which had been calibrated previously by using a cell counting chamber (Hemacytometer Set, Hausser Scientific).

5.4.6.2 Bioluminescence of Bacteria in Planktonic Form

Porous packs (PPs) each consisting of a 1 cm thick cell strainer (BD Falcon™ 352360) with 100 µm nylon mesh and filled with dried glass beads were used to determine the intensity of planktonic cell bioluminescence in porous medium (Figure 5-1). To
accomplish this, a single PP was placed in a 60 cm Petri dish filled with induction medium containing a known population density of HK44 cells (determined spectrophotometrically). Each Petri dish contained a different concentration of cells. A bioluminescence image of the PP in each Petri dish was then recorded (Figure 5-1) and used to develop a correlation between recorded bioluminescence intensity and cell density. By simultaneously evaluating the difference in bioluminescence between planktonic cells in the PP and planktonic cells in another region of the same dish where there was no porous medium present, the effect of porous medium on bacterial bioluminescence intensities could be determined.

Figure 5-1: (A) Porous Package (PP) used to evaluate bacterial bioluminescence in porous media and liquid culture. Each PP was consisted of a cell strainer, filled with glass beads and placed in a Petri dish containing growth or induction medium. B) Bioluminescence image of a PP in a Petri dish. Simultaneous evaluation of planktonic cells bioluminescence in porous medium and planktonic cells in liquid culture in the same Petri dish allowed for a determination of the effect of porous medium on bacterial bioluminescence intensities.
5.4.6.3 Bioluminescence of Bacteria in Biofilm Form

To determine bioluminescence activity of biofilm bacteria in static culture, 25 PPs were inoculated by immersing each PP in cell inoculum (6\times10^5 cells/mL) for 8 hours to provide sufficient time for bacterial attachment to the porous matrix. Subsequently, each PP was placed by itself in Petri dish containing 28 mL of growth medium to allow for bacterial proliferation and formation of biofilm in porous medium. Every 12 hours, one of the PPs was transferred to a different Petri dish containing fresh induction medium, and after measuring the bioluminescence being emitted from the biofilm, the bacterial cell density in that PP was measured (see Section 5.4.8 for details).

In addition, bacterial cells were allowed to grow and form biofilm under flow conditions in a saturated porous medium to simultaneously evaluate biofilm bioluminescence and its effects of hydraulic conductivity. To accomplish this, packed tubes (PTs), each consisting of a rectangular clear acrylic tube (0.95 cm internal diameter) filled to a height of 2 cm with dried glass beads, were used to grow biofilm under a constant pressure gradient. The beads were kept in place by 0.1 mm nylon mesh screens at the inlet and outlet (Figure 5-2). Each PT was filled with sterile growth medium via a peristaltic pump (Gilson Minipuls 3) while orientated in a vertical position. The same pump was then used to add 0.6 mL of cell culture inoculum (6\times10^5 cells/mL) to each PT. We previously verified that the pump did not adversely impact bacterial cell viability. Bacteria were allowed to attach to the glass beads for 18 hours and then continuous flow of growth medium was initiated using a Mariotte tube (SMS®, Arizona, USA) to maintain a constant pressure head. At regular intervals, each PT was simultaneously evaluated for microbial bioluminescence.
Figure 5.2: Packed tubes (PT), each consisting of 4 rectangular clear acrylic tubes (0.95 cm internal diameter) filled to a height of 2 cm with dried glass beads. PTs were used to grow biofilm under a constant pressure gradient and study hydraulic conductivity variations.

and fluid flow rate. The flow rate was recorded using a calibrated in-line flowmeter (BEL-ART Riteflow®) at the outlet of the chamber. This enabled the hydraulic conductivity of the porous medium to be determined using the equation:

\[ K = \frac{qL}{Ah} \]  

(5.2)

where \( q \) is volumetric flow rate of the water phase, \( A \) is cross-sectional area of the porous medium, \( K \) is hydraulic conductivity, and \( h \) is hydraulic head difference applied over a length \( L \). During the induction phase, growth medium was replaced by induction medium to induce the lux-genes.
5.4.7 Gravimetric Measurements

Biofilm density was evaluated gravimetrically. By assuming that biofilms are composed of bacteria, EPS, and interstitial water, biofilm density is given by:

\[
\rho_b = \left( \frac{m_{db}}{\epsilon_s} + \epsilon_v V_b \rho_w \right)/V_b
\]  \hspace{1cm} (5.3)

where \(\rho_b\) is biofilm density, \(m_{db}\) is the mass of dried biofilm, \(\epsilon_s\) is solid mass fraction of biomass (i.e. dry mass of biomass per total mass of biomass), \(\epsilon_v\) is biofilm void fraction (i.e. biofilm water content), \(V_b\) is volume of biofilm, and \(\rho_w\) is the density of water. However, biofilm dry density, defined as the mass of dry biofilm per unit hydrated biofilm volume (biofilm volume including water in biofilm), can be calculated by determining the hydrated biofilm volume and the mass of dried biofilm after removing all water content. Therefore, the actual biofilm volume can be estimated by measuring the mass of dried biofilm using the equation:

\[
V_b = \frac{m_{bd}}{\rho_{bd}}
\]  \hspace{1cm} (5.4)

where \(\rho_{bd}\) is the biofilm dry density. Thus, for gravimetric measurements, bacterial cells were placed in growth medium (3 replicates) and allowed to form biofilm on glass slides (Figure 5-3). After 12 days, the biofilm thickness (\(L_b\)), was measured with an optical microscope (Axio Observer.A1, Zeiss, Germany) according to the method of Bakke and Olsson (1986).
Figure 5-3: Biofilm volume was estimated from high resolution images of biofilm coated glass slides. Bacterial cells were allowed to attach and form biofilm for 12 days on the glass slide and subsequently, biofilm thickness and surface area of the covered glass slide was measured.

Nine optical-thickness measurements were taken at regular intervals for each slide and then averaged to obtain a mean biofilm thickness on that slide. However, measured optical thicknesses do not represent the actual physical thickness of biofilm due to the different reactive indices of air and biofilm. Therefore, as stated by Bakke and Olsson (1986), a correction factor of 4/3 should be applied to evaluate bacterial biofilm thickness. Also, surface coverage was estimated by image analysis from digital images taken using the previously described imaging system. Using MATLAB® Image Processing Toolbox (MATLAB® R2009a, MathWorks Inc.), a threshold value (Otsu,
1979) was applied to biofilm images so that those areas covered with biofilm appeared white, while the remaining area appeared black. The relative surface coverage of the biofilm was then calculated as the proportion of white to the total area. This thresholding approach has been commonly used with images of biofilm to discern the biological component from background (Stoodley et al., 1998; Bozorg et al., 2012).

The slides were then dried at 85°C for 24 h and after being weighed, the dried biofilm was washed off with de-ionized water. The clean slides were dried for 24 hours, examined to ensure all biofilm had been removed, and then weighed again. The biofilm dry mass ($m_{db}$) could be calculated from weight difference of the slides with and without dried biofilm. The biofilm dry density was then found by using Equation 5.4.

Since identical growth conditions were used to grow biofilm in porous media, the same dry density was assumed for the biofilm formed in the fully saturated porous media (Characklis and Cooksey, 1983). Therefore, to estimate the biofilm volume, each PP was weighed prior to inoculation. After inoculation and at different time intervals, inoculated PPs were removed from the Petri dishes, dried at 85°C for 24 hours and weighed to determine dry mass of biofilm. By using the measured biofilm dry mass and biofilm dry density, the fraction of pore space occupied by biofilm (i.e. biofilm saturation) was calculated.

**5.4.8 Bacterial Cell Enumeration**

At the end of an experiment, biofilm covered porous media, either in PPs or PTs, were placed into separate test tubes, each containing a known volume of PBS, and vortexed and sonicated repeatedly to detach biofilm from glass beads (Heersink, 2003) (Appendix
C). These samples were used to determine the bacterial cell numbers by measuring the total DNA weight (Qubit® dsDNA Assay Kit, Invitrogen, Oregon, USA) (please see Appendix F for details). Since a single cell strain was used, the total DNA weight could be used to evaluate bacterial concentration. Total mass of HK44 DNA was calculated to be 7.843x10^{-15} g per cell based on the PUTK21 plasmid used in HK44 and DNA of the natural bacteria (King et al., 1990). A standard curve, correlating mass of DNA to bacterial cell population size, was generated by manual cell counting in a counting chamber (Hemacytometer Set, Hausser Scientific) (Appendix G). Cell numbers (at least 360 bacteria per slide for statistical relevance) were counted in triplicate for each sample.

5.5 Results and Discussions

5.5.1 Oxygen Effects

Lux gene dependent bioluminescence has been shown to be an aerobic process (Meighen and Dunlap, 1993; Uesugi et al., 2001). Hypoxia, therefore, leads to a decrease in bioluminescence. The kinetic values for these processes have been studied extensively for both immobilized (Webb et al., 1997) and suspended (Kelly et al., 2003) cells. Thus, prior to investigating any application of lux gene dependent bioluminescence, it was deemed important to first determine the oxygen levels needed to make sure it did not limit bacterial bioluminescence during subsequent experiments. To accomplish this, planktonic cultures of HK44 were inoculated into induction medium which had been aerated with an aquarium air pump (Whisper® 196 Tetra Holding, US). The vessel was operated in batch mode and oxygen concentration and bioluminescence was
simultaneously measured versus time. As shown in Figure 5-4, bioluminescence was not detected for approximately the first 20 minutes. During this period, there was not much change in oxygen concentration indicating that the cell oxygen uptake rates were minimal. However, after 20 minutes, there was a rapid rise in bioluminescence for approximately 45 minutes, coinciding with a higher oxygen uptake rate. These observations were consistent with other experimental studies (Uesugi et al., 2001). Also as expected, our results revealed that low oxygen concentrations negatively affect bioluminescence levels (Figure 5-4).

Figure 5-4: Effect of oxygen saturation on bioluminescence intensity of planktonic cells of HK44 in induction medium which had been saturated with sterile air. Experiment conducted in batch mode, and the oxygen concentration and bioluminescence levels were simultaneously determined over time.
To quantify this impact and determine oxygen consumption rates during maximum bacterial bioluminescence, evolving bioluminescence activity and changes in oxygen concentration were monitored in planktonic cultures of HK44 in fully aerated (Whisper® 196 Tetra Holding, US) induction medium (Figure 5-4). The same trends were observed for cell densities ranging from $1 \times 10^5$ to $2 \times 10^8$ cells/mL. The results revealed that depending on bacterial cell population density, bioluminescence intensities increased up to a maximum value, remained constant for a period of time (e.g. 42 minutes for a density of $2 \times 10^8$ cells/mL), and then exhibited a sharp drop after reaching a critical dissolved oxygen concentration.

As shown in Figure 5-4, stable oxygen consumption rates during maximum bacterial bioluminescence, and the corresponding critical oxygen concentration (e.g. 6 µmol/min and 50 µmol oxygen, respectively, for a density of $2 \times 10^8$ cells/mL) implies that higher oxygen concentration could potentially enlarge the bioluminescence period. Further investigations revealed that saturating the medium with pure oxygen significantly increases the constant bioluminescence period by up to 250 minutes while keeping the maximum bioluminescence value intact (Figure 5-5). Therefore, to reduce the negative impact that oxygen availability had on bacterial bioluminescence, all media were saturated with pure oxygen (Praxair Inc.) for all remaining experiments.
Figure 5-5: Bioluminescence response of planktonic cultures of HK44 with different population densities in induction medium which had been fully saturated with pure oxygen. Higher oxygen saturation levels significantly increased the constant bioluminescence period by up to 360 minutes while keeping the maximum bioluminescence value unchanged.

5.5.2 Cell Density Evaluation

Figure 5-6 shows applicability of lux gene dependent bioluminescence to evaluate bacterial cell densities. More than 70 samples with different cell densities were studied to obtain a relationship between bioluminescence activity of planktonic cells in liquid culture and in porous medium. As shown in Figure 5-6, the bioluminescence detected for planktonic cells within the porous medium was lower compared to the other regions of the Petri dish (i.e. liquid culture) which could be related, in part, to the lower number of cells per unit volume of porous medium compared to liquid culture, as well as the shielding of emitted bioluminescence by the particles making up the porous medium.
Figure 5-6: Bioluminescence activity of planktonic cells in liquid culture and in porous medium indicating that the detected bioluminescence intensity of planktonic cells within the porous medium was lower than that exhibited by planktonic cells in liquid culture. Results indicated the applicability of lux gene dependent bioluminescence in evaluation of planktonic bacterial cell densities in liquid culture.

Figure 5-7: Bioluminescence activity of biofilm cells. Based on the cell densities, lower bioluminescence activity was observed for the biofilm cells in porous medium.
Also, based on cell densities, even less bioluminescence activity was observed for the biofilm cells, as shown in Figure 5-7. In biofilms, microorganisms are enclosed in their self-produced EPS. Therefore in porous structures, the results demonstrate that the bioluminescence intensity of biofilm cells was not only affected by the porous medium, but also could be influenced by microbe secreted EPS matrix. However, according to Figure 5-8, all three sets of data revealed a power relationship between cell density and detected bioluminescence intensity. Furthermore, regression of the data revealed that all the power functions possessed similar exponents, all equal to about 3.2, with different scaling factors, implying that bioluminescence exhibits the same behavior with respect to cell populations in all 3 sets of data while the scaling factors indicate environmental effects on the detected bioluminescence intensities.

Figure 5-8: Regression analysis of data revealed a power relationship between cell density and detected bioluminescence intensity in all three sets of data for planktonic bacteria in liquid culture, planktonic bacteria in porous media, and biofilm bacteria in porous media.
Such nonlinear relationships between metabolic activity (e.g. bacterial bioluminescence) and the number of cells have been observed in many biological processes, such as metabolic rates of entire organisms, maximal population growth rate, concentration of metabolic enzymes, and sizes of biological structures, with allometric scaling exponents ranging in value from 1.3 to 4 (West and Brown, 2005).

5.5.3 Biofilm Saturation

Based on the measured biofilm thickness (Figure 5-3), area of covered surface, and dry weight of biofilm, biofilm dry density was calculated to be equal to 42.1 mg/mL which is consistent with values of 30 to 80 mg/mL reported in the literature (Beyenal et al., 1998; Brito and Melo, 1999; Casey et al., 2000; Beyenal and Lewandowski, 2002; Garcia Lopez et al., 2003). Based upon the obtained dry biofilm density, the weight difference between each dried PP and its original (pre-inoculation) weight was used to determine biofilm volume in the porous medium. In addition, prior to drying, the bioluminescence intensity of each PP was recorded. As shown in Figure 5-9, a power law provides a good fit between the calculated biofilm saturation and bioluminescence intensity. Under similar growth and induction conditions, this power law was used to estimate biofilm saturation in porous medium in the other experiments.
Figure 5-9: Using biofilm dry density, biofilm saturation was evaluated gravimetrically in porous packages. The results revealed that a power function provides a good fit between calculated biofilm saturation and recorded bioluminescence intensity. Scale indicates increasing bioluminescence intensity from 0 to 16.

5.5.4 Hydraulic Conductivity

An increase of the biofilm saturation in porous medium reduces the void space available for fluid flow, and thus, lowers hydraulic conductivity. In the present study, significant reductions in hydraulic conductivities were observed during the biofilm growth in PTs (Figure 5-10), consistent with other reports in the literature (Cunningham et al., 1991, 2003; Lappin-Scott et al., 1988; Bielefeldt et al., 2002; Seki et al., 2006; Bozorg et al., 2012). Considering the relationship observed between biofilm characteristics
Figure 5-10: Hydraulic conductivity of porous media versus bioluminescence intensity of each packed tube showing an exponential correlation.

(i.e. bacterial cell population and biofilm saturation), and bioluminescence intensity (Figures 5-6, 5-7, 5-8, and 5-9), it could be suggested that bacterial bioluminescence may also be used in real-time evaluation of porous medium hydraulic conductivity. To verify if such relationship is valid, Equation 5.2 was used to determine hydraulic conductivity of porous medium based on the applied pressure head and the fluid flux through the PTs. Subsequently, relative hydraulic conductivities (calculated as the ratio of the effective hydraulic conductivity divided by the saturated hydraulic conductivity of the clean porous medium) were plotted against bioluminescence intensity of the induced cells, shown in Figure 5-10, which were evaluated by injection of induction medium to the PTs immediately after the flux determination. According to Figure 5-10, the relationship between hydraulic conductivity and bioluminescence intensity appears to follow an
exponential trend, thereby suggesting that bacterial bioluminescence can be used to directly estimate porous medium hydraulic conductivity.

Hydraulic conductivity has previously been reported to be exponentially related to porosity (Sahimi, 1995; Clement et al., 1996). Therefore, by assuming that the total porosity, $\varphi$, consists of a void porosity, $\varepsilon_v$, and an occupied biofilm fraction, several authors (Ives and Pienvichitr, 1965; Okubo and Matsumoto, 1979; Knapp et al., 1988; Clement et al., 1996) proposed the following relationship:

$$\frac{K}{K_{\text{initial}}} = \left(\frac{\varepsilon_v}{\varphi}\right)^n \quad n > 0$$

(5.5)

where $K_{\text{initial}}$ is initial (clean) hydraulic conductivity and $n$ is an exponent that varies between different models based on the microgeometrical properties of the porous medium and the morphology of the biofilm. By assuming a homogenous and uniform biofilm growth on solid particles, Clement et al. (1996) developed a relationship identical to Equation 5.5 and found that for typical sandy materials, $n$ had a value of 19/6.

By using the correlation between bioluminescence intensity and saturation of biofilm previously determined in PPs, relative hydraulic conductivities could be obtained as a function of biofilm saturation (Figure 5-11). Obtained results revealed that low biofilm saturation (less than 4%) has negligible effects on porous medium hydraulic conductivity; however, elevated values can significantly alter porous medium hydraulic properties.
Figure 5-11: Using the bioluminesce of bacterial cells, evolving biofilm saturation under flow conditions were assessed in packed tubes and plotted against relative hydraulic conductivity. The results indicate that the published Clement model (dashed line) tends to overestimate hydraulic conductivities at similar biofilm saturation levels. However, an expression equivalent to the Clement model but with a higher exponent ($n=6.3$) provides a good fit.

Experimental results were also compared with the Clement model, a theoretical model which has been used extensively in published bioclogging studies (Thullner et al., 2002; 2004; Brovelli et al., 2008), to see whether estimated biofilm saturation and measured hydraulic conductivity follow theoretical predictions. As shown in Figure 5-11, the results reveal that the Clement model may overestimate hydraulic conductivities at similar biofilm saturations. This can be explained by the fact that the Clement model ignores the possibility of pore throat clogging even though this may have significant effects on hydraulic conductivity variations.
However, although the experimental data were not consistent with the Clement model, we found that an expression equivalent to Equation 5.5, with $n=6.33$, could be used to predict interactions between hydraulic conductivity and biofilm saturation at elevated biofilm saturations. Higher exponent values in the bioclogging model (Equation 5.5) correspond to the fact that even small changes in porosity can cause severe hydraulic conductivity reductions. This would happen when pore connections have been closed due to clogging of the pore throats.

5.6 Conclusion

The development of methods to nondestructively evaluate the impact of microbial biofilm formation on fluid flow through a porous medium would be a significant advancement which could ultimately help to better understand and control industrially relevant processes such as filtration and bioremediation. We have shown here that it is possible to evaluate cell density and hydraulic conductivity within a porous medium by simply measuring the bioluminescence intensity of a model bioluminescent bacterium. Biofilm saturation in porous medium was also related to the bioluminescence intensity, thereby allowing the development of an expression for relative hydraulic conductivity. The correlations developed in this work can be used to noninvasively determine, in real-time, cell density, biofilm saturation, and hydraulic conductivity within porous structures. In addition to better understanding how microbial biofilms impact flow processes in porous media, this work will facilitate simulation studies which could serve to better predict, and possibly even control, biofilm behavior in porous media.
Chapter 6

Effect of Extracellular Polymeric Substances on
Bacterial Fate and Transport in Porous Media

6.1 Preface
The present thesis is based on refereed papers that have already been published or are in
the process of publication. Unavoidably, there is some repetition between this chapter and
Chapters 4 and 5, particularly in the Introduction section where biofilm and its
applications are introduced, as well as in the Materials and Methods, where experimental
procedures are described. Therefore, in this chapter, sections of Introduction and
Materials and Methods may be safely skipped without loss of coherence. In addition,
more comprehensive details of experimental procedures used to prepare culture media,
sample the porous media, prepare biofilm samples, and determine bacterial population, as
well as the assays used to measure bacterial DNA and protein concentration are provided
in Appendices A through G. Also, MATLAB codes used to process the bioluminescence
images are summarized in Appendix J.
6.2 Abstract

According to the DLVO theory, named after its inventors Derjaguin, Landau, Verwey, and Overbeek, particle transport and removal in physicochemical colloidal filtration is governed by electrostatic and van der Waals interactions. However, in the case of bacterial transport in porous media, extracellular polymeric substances (EPS) play an important role in bacterial adhesion to porous matrix by altering the hydrophobicity of contact surfaces. EPS are also associated with irreversible adhesion of bacteria. Surface attached bacteria can establish a thin layer of biofilm on solid surfaces by excretion of EPS, enhancing further bacterial adhesion and colonization. Therefore, as hydrophobic interactions play an important role, an extended DLVO theory, in which the acid-base (hydrophobic) interactions are included, will enable interpretation of EPS affected porous media removal efficiency. According to colloidal filtration theory, the removal efficiency of a filter medium is characterized by two main factors: collision efficiency and sticking efficiency. Biofilm development can significantly influence both parameters in a porous medium and thus, affect the removal efficiency of that filter medium. However, polymeric interactions in bacterial adhesion are not well understood and a method to calculate such interactions is not available yet. Here, by using colloidal filtration theory, polymer interactions were quantified and bacterial sticking efficiency of the biofilm coated particles was calculated.
6.3 Introduction

The formation of a bacterial biofilm starts when planktonic bacteria attach to a solid surface in a moist environment (Figure 6-1). Subsequent cell division coupled with the simultaneous secretion of adhesive extracellular polymeric substances (EPS) causes the generation of a biofilm monolayer on the solid surface (van Loosdrecht et al., 1990). Once the monolayer of biofilm is established, further cell duplication and EPS production results in biofilm maturation (Kober et al., 1994). In porous media, which inherently have large surface area to volume ratios, planktonic bacteria being transported by a fluid flowing through the pores can attach to and colonize the surface of the porous matrix, thereby initiating biofilm formation.

Whereas biofilms have typically been viewed as undesirable due to their significant negative impact on the physicochemical and geohydrological properties of a porous medium, it is conceivable that better understanding their formation and maturation may actually facilitate better control of these biological entities, thereby enabling the development of highly efficient biofilm based processes involving porous structures. For example, biofilms could be tailored to have utility in a variety of environmental and industrial applications, including protection of groundwater from pathogen migration (Abuashour et al., 1994), *in situ* subsurface bioremediation (Gross and Logan, 1995), water and wastewater treatment (Waybrant et al., 1998; Urfer et al., 1997; Golab et al., 2006; Davis et al., 2007), and enhanced oil recovery (Bass and Lappin-Scott, 1997; van Hamme et al., 2003). The design and field scale implementation of such applications will require a full understanding of each step of the biofilm development process under flow conditions, starting with bacterial adhesion under the particular physical, geochemical,
Figure 6-1: Stages in bacterial biofilm development: A) planktonic bacterial cells contact the solid surface; B) based on the contact angle and surface properties, some bacteria may reversibly attach to the solid surface; C) reversibly attached bacteria grow and proliferate through by cell duplication in the presence of sufficient nutrients; D) bacteria attach irreversibly by secretion of EPS and form a thin layer of biofilm; E) preliminary steps of biofilm maturation by initiating development of biofilm structure; and F) second maturation (fully mature biofilm) indicated by the complex biofilm structure and architecture.

and biological conditions found in porous media. Despite the numerous biofilm-based applications currently in use, there is a lack in the mechanistic understanding of processes affecting transport and deposition behavior of bacteria in granular porous media, the available theories are arguably incomplete, and evaluation of such theoretical models against laboratory or field experimental data are few (Gerlach and Cunningham, 2010).
By considering bacteria as colloidal particles, the classical colloid filtration theory (CFT) has been used to study bacterial transport and fate in porous media. As described in CFT, adhesion of colloidal particles to the solid surface of a collector is controlled by mass transfer of suspended particles from the bulk flow to the surface of the grains (i.e. collectors) making up the medium (Yao et al., 1971; Li and Logan, 2004). Once a particle strikes the collector surface, interactions with the solid surface govern whether or not the particle will attach. The classical DLVO theory (Derjaguin et al., 1941; Verwey et al., 1948) has been used extensively to describe such interactions, by considering the net energy of interactions, including attractive van der Waals forces and generally repulsive electrostatic forces (Rutter and Vincent, 1980).

However, it could be argued that DLVO theory expresses only one of several mechanisms of the adhesion process and ignores various molecular interactions that are present in the presence of polymeric substances on striking surfaces. Thus, to better elucidate diverse colloidal attachment behaviors, DLVO theory was later extended (van Oss et al., 1986; van Oss 1994) by inclusion of short-range acid-base interactions (which account for the hydrophobicity of the striking surfaces) and revealed that hydrophobicity of surfaces is a major determinant of colloidal attachment (Azeredo et al., 1999). In biological processes, the application of DLVO type theories has not been successful for the elucidation of diverse bacterial attachment behaviors (Azeredo et al., 1999; Parent and Velegol, 2004). Based on DLVO theory, bacteria, as colloidal particles with smooth rigid surfaces, adhere when they are close enough to a surface to overcome electrostatic repulsive forces. Obviously, this single mechanism does not explain various molecular interactions influencing bacterial adhesion especially, when bacterial surface
exopolymers interact with macromolecular groups on the substratum and thus, interfere with DLVO type interactions (Ong et al., 1999; Camesano and Logan, 2000).

It has been postulated that increased bacterial adhesion to solid surfaces is related to the adsorption of the outer membrane polymers to the solid surfaces (Burks et al., 2003; Lytle et al., 2002). Bacteria are mostly able to excrete polymeric compounds which can be associated with the cell wall or be released into the medium (Decho et al., 2005). The higher affinity of such exopolymers to the surface compared to the aqueous phase facilitates bacterial adhesion by two main processes: surface modification (polymer attraction between bacterial and surface polymers resulting in improved sticking efficiency) (Figure 6-2A) and surface enhancement (occupying higher portion of available void space to floating cells leading to higher collision efficiency) (Figure 6-2B).

![Figure 6-2: Presence of EPS on solid surfaces enhances bacterial attachment by: A) changing solid surface physicochemical properties (hydrophobic interactions) and B) increasing surface area for bacterial deposition (reducing available pore space and/or bridging the pore throats).](image-url)
Researchers have studied bacterial cell surface characteristics, such as surface free energy (Busscher et al., 1984) surface charges (Gannon et al., 1991), surface hydrophobicity (Gannon et al., 1991; James, 1991), and surface polymers that are involved in bacterial attachment (Williams and Fletcher, 1995). However, properties of the solid collector surface can also have significant impact on the attachment process. Numerous studies have investigated the influence of collector surface properties on bacterial removal efficiency (i.e. fraction of the bacteria remained in porous media), including surface roughness (Li and Logan, 1999; Scheureman et al., 1998), collector sizes (i.e. solid surface area) (Fontes et al., 1991; Guber et al., 2007; Jeng et al., 2005; Bengtsson and Ekere, 2001), surface charge (Gottenbos et al., 1999), chemical composition of solid material (Cordero et al., 1996; Gottenbos et al., 2000; Tegoulia and Cooper, 2002; Buczynski et al., 2003; Henriques et al., 2004; Speranza et al., 2004) , and hydrophobicily (Balazs et al., 2003).

In the case of bacterial transport in porous media, following bacterial attachment, EPS is synthesized by the attached cells to strengthen their binding to the solid substratum. Secretion of EPS at initial stages results in formation of a thin biofilm layer which can significantly modify physicochemical properties of solid surfaces, thereby altering porous medium bacterial removal efficiency. Therefore, unlike colloidal filtration, bacterial removal in porous media should be considered as a dynamic process in which intrinsic collectors properties in the filter medium can be modified following bacterial adhesion due to the biological activity of living organisms.

In this study, transport and fate of a gram negative bacterium, *Pseudomonas fluorescens*, was investigated in liquid-saturated granular packed-beds. Porous media with four
different sizes of solid particles were used to evaluate the effect of collector sizes on packed-bed bacterial removal efficiency. Furthermore, biofilm mediated removal efficiencies in each packed-bed was investigated by coating the porous media by a thin layer of biofilm (i.e. surface attached cells embedded in self produced EPS). Biofilm affected attachment coefficients were calculated and compared with that in clean packed-beds and the outcomes were interpreted in terms of the DLVO and extended DLVO theories.

6.4 Materials and Methods

6.4.1 Bacterial strain

The biofilm forming bacterial strain used in this study was the bioluminescent reporter strain *Pseudomonas fluorescens* HK44 (hereafter referred to HK44) obtained from the University of Tennessee, Center for Environmental Technology (University of Tennessee, Knoxville, TN). This strain has been genetically engineered by insertion of the salicylate-inducible *luxCDABE* gene cassette and a tetracycline resistance marker and thus, luminesces in the presence of aromatic hydrocarbons such as naphthalene, salycylate, and 4-methyl salicylate (King et al., 1990). It has been shown that detected bioluminescence light intensity can be used to evaluate bacterial cell density and biofilm saturation (i.e. the pore volume fraction occupied by biofilm) (Chapters 4 and 5).

6.4.2 Culture Preparation

All glassware and materials used in the experiments were sterilized by autoclaving at 121°C and 15 psi for 20 minutes. The growth medium used to expand and maintain the bacterial population in liquid cultures was an oxygen-saturated, nitrate-free growth
medium (Appendix A) consisting of MgSO\(_4\) (0.4 g/L); CaCl\(_2\).2H\(_2\)O (0.1 g/L); NH\(_4\)Cl (0.4 g/L); NaCl (8 g/L); KCl (0.2 g/L); NaH\(_2\)PO\(_4\) (1.15 g/L); K\(_2\)HPO\(_4\) (0.26 g/L); HCl (0.00366 g/L); FeSO\(_4\).7H\(_2\)O (0.021 g/L); H\(_3\)BO\(_3\) (0.0003 g/L); MnCl\(_2\).4H\(_2\)O (0.001 g/L); CoCl\(_2\).6H\(_2\)O (0.0019 g/L); NiCl\(_2\).6H\(_2\)O (0.00024 g/L); CuCl\(_2\).2H\(_2\)O (0.00002 g/L); Na\(_2\)EDTA.2H\(_2\)O (0.01 g/L); ZnSO\(_4\).7H\(_2\)O (0.00144 g/L); Na\(_2\)MoO\(_4\).2H\(_2\)O (0.00036 g/L), supplemented with 1.0 g/L glucose as the main carbon source. The pH of all stock solutions was adjusted to 7.20 ± 0.05 using 1.0 M NaOH. Also, HK44 contains a tetracycline resistance marker. Thus, to ensure plasmid maintenance, tetracycline solution (EMD Chemicals, OmniPur® EM-8990) was added to autoclaved media (30 mg/L final concentration) via a 0.2 μm filter. In addition, an induction medium was prepared to induce luminescence, by adding 0.1 g/L (final concentration) of salicylate to induce the lux genes. Note that all phosphate sources were removed from the induction medium to restrict HK44 proliferation while keeping metabolic activities intact (Webb et al., 1997; Oates et al., 2005; Bozorg et al., 2012).

Frozen cultures of HK44 were recovered routinely using Luria broth (LB) medium in a rotary shaker (Heidolph Unimax 2010, Germany) set at 150 rpm. Following overnight incubation, 0.5 mL of the culture was inoculated into 40 mL of fresh growth medium. After 24 hours, cells were harvested by centrifugation (Beckman Coulter®, X-22R) at 10,000 rpm for 20 min, washed in PBS, centrifuged again and resuspended in induction medium to a density of 5x10\(^8\) cells/mL (OD\(_{550}\) of approximately 0.35 – Appendix E) to confirm bioluminescence activity. This bacterial suspension was used to conduct the column experiments (described below).
6.4.3 Porous Media

In this study, 4 different sized translucent acid-washed glass beads (Sigma–Aldrich, G1277, G8772, Z273619, and Z273627), with average particle diameters of 250, 500, 1000, and 2000 μm were used as solid collectors to determine the effect of collector sizes on packed bed bacterial removal efficiency. Porosities of the packed beds were calculated gravimetrically by determining the volume of glass beads and water in the dry-packed and saturated wet-packed porous media (Table 6-1). In addition, in order to investigate

Table 6-1: Experimental conditions for breakthrough experiments and parameters used to study bacterial attachment in porous media.

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Packed-Bed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Collector Diameter (dc)</td>
<td>mm</td>
<td>0.25±0.044</td>
</tr>
<tr>
<td>Sieve Size</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>Specific Surface Area (a)</td>
<td>1/cm</td>
<td>133.92</td>
</tr>
<tr>
<td>Cross Sectional Area (A)</td>
<td>cm²</td>
<td>2.25</td>
</tr>
<tr>
<td>Length (L)</td>
<td>cm</td>
<td>10</td>
</tr>
<tr>
<td>Porosity (φ)</td>
<td>-</td>
<td>0.442</td>
</tr>
<tr>
<td>Temperature (T)</td>
<td>K</td>
<td>298</td>
</tr>
<tr>
<td>Fluid Density (ρ_w)</td>
<td>g/cm³</td>
<td>1.05</td>
</tr>
<tr>
<td>Fluid Viscosity (μ_w)</td>
<td>g/cms</td>
<td>9×10⁻³</td>
</tr>
<tr>
<td>Hamaker Constant (H)</td>
<td>J</td>
<td>6.5×10⁻²¹</td>
</tr>
<tr>
<td>Bacterial Diameter (d_pb)</td>
<td>mm</td>
<td>1.44×10⁻³</td>
</tr>
<tr>
<td>Bacterial Density (ρ_pb)</td>
<td>g/cm³</td>
<td>1.105</td>
</tr>
<tr>
<td>Boltzman Constant (K_B)</td>
<td>J/K</td>
<td>1.38×10⁻²³</td>
</tr>
</tbody>
</table>
biofilm mediated packed-bed removal efficiency, glass beads were inoculated by HK44 suspension (5x10^7 cells per mL) in growth medium and incubated for 24 hours at 25 °C. This incubation period in batch culture provided sufficient time for bacteria to irreversibly attach to the glass beads by producing EPS and thus, cover the glass beads by a thin layer of biofilm.

6.4.4 Column Experiments

To study bacterial fate and transport in porous media, column experiments were conducted using square clear acrylic tubes with inner dimension of 1.5 cm (AST0004, Industrial Plastics and Paints) filled to two different heights for fine (250 and 500 μm glass beads) and coarse (1 and 2 mm glass beads) collectors (Figure 6-3) (please see Table 6-1 for more information). Due to the low removal efficiencies measured in the packed-beds with coarse collectors, column lengths were doubled in those experiments to better measure bacterial concentrations in column effluents. However, according to the elevated pressure drops observed in columns with fine collectors, lengths of those columns were kept unchanged. Calculated parameters, in any case, were reported per unit length of the packed-beds. Furthermore, by keeping the operating conditions and column characteristics identical in packed-beds of similar collector sizes, removal efficiencies could be compared in terms of the collector surface properties in clean and biofilm coated packed-beds.

Both ends of the columns were sealed with PVC caps equipped with plastic adaptors to inject and collect liquid media. Columns were packed by slow pouring glass beads into the column containing a pore volume (PV) of sterile de-ionized water, while periodically
Figure 6-3: Clear square acrylic tubes with inner diameter of 1.5 cm were used to conduct column experiments. Columns were filled up to 10 and 20 cm for fine (250 and 500 μm) and coarse (1 and 2 mm) glass beads.
tapping to release any trapped air bubbles. Glass beads (i.e. collectors) were retained in the column by an aluminum mesh placed over the bottom PVC cap. A small portion of the column was left unpacked, in order to make a thin stationary layer of liquid at the surface of porous media to ensure that any injected fluid was equally distributed across the cross-section of the column during the experiments. A tracer solution (Brilliant Blue FCF dye, ACROS Organics) was then introduced into the packed column, using a Mariotte tube to maintain constant pressure head, to obtain breakthrough curves (BTCs) of the conservative tracer (i.e. no adsorption of tracer) in each porous medium. Subsequently, the observed BTCs were imported to the studio of analytical models package (STANMOD) (Simunek et al., 2007) to estimate porous media dispersivity. The STANMOD software suite is a combination of different widely used analytical solutions which can be used to approximate parameters of one, two, or multidimensional solute transport problems in porous media. Here, a modified version of CXTFIT code (Toride et al., 1999), which is included in STANMOD package, was used to solve the inverse problem by fitting the obtained experimental results of BTCs to mathematical solutions of theoretical one-dimensional transport models. In addition, fluid flowrates were recorded during tracer experiments by using a calibrated in-line flowmeter (BEL-ART Riteflow®) to calculate hydraulic conductivity of each packed bed.

Following the tracer experiment, the column inlet was switched to a variable flow peristaltic pump (Gilson Minipuls 3), and downward flow of sterile induction medium was regulated through the column. Once steady state flow conditions (equal to one PV per hour for each column) was established, 1 PV pulse of bacterial suspension in induction medium was pumped through each porous medium by switching the influent to
the cell suspension reservoir, followed by elution with 6 PVs of sterile induction medium. During the experiments, effluent samples were collected every 0.1 pore volume and analyzed for bacterial population.

6.4.5 Bioluminescence Imaging

A 14-bit CCD camera (Progres MFcool, Jenoptik, Germany) with interline 2/3" Sony ICX285AL 1.4 megapixel progressive scan monochrome CCD sensor (encased by a hermetically sealed, nitrogen-flushed capsule, cooled by a Peltier element, a heat sink and a fan) equipped with a f/1.4 focal ratio Computar Megapixel lens was used to capture grayscale bioluminescence images in a dark box. CapturePro software (ProgRes® CapturePro 2.7.7, Jenoptik, Germany) was used to control and program the CCD camera. The imaging process and correlation of bioluminescence intensity to biological activity that were used in this study were previously validated and are described extensively in Chapter 4.

6.4.6 Bacterial Enumeration

Bacterial cell densities in suspension were determined by measuring the total DNA weight (Qubit® dsDNA Assay Kit, Invitrogen, Oregon, USA). Since a single cell strain was used, the total DNA weight could be related to the bacterial concentration. The total mass of HK44 DNA was calculated to be $7.843 \times 10^{-15}$ g per cell based on the PUTK21 plasmid used in HK44 and DNA of the natural bacteria (King et al., 1990). A standard curve, correlating mass of DNA to bacterial cell population size, was also generated by manual cell counting in a counting chamber (Hemacytometer Set, Hausser Scientific) (Appendix G). Cell numbers (at least 360 bacteria per slide for statistical relevance) were counted in triplicate for each sample.
6.4.7 Model Development

Different processes govern movement and fate of bacteria in saturated porous media which can be classified into two major groups: (i) transport, which is mainly governed by convection/dispersion mechanisms and (ii) exchange between liquid suspension and solid substratum (i.e. bacterial adhesion). Therefore, by assuming irreversible attachment, governing equations for bacterial transport and fate in saturated porous media can be described as (Harvey and Garabedian, 1991; Hornbergeer et al., 1992; McCaulou et al., 1995):

\[
\frac{\partial C_{pb}}{\partial t} + \lambda_{att} C_{pb} = D \nabla^2 C_{pb} - u \cdot \nabla C_{pb} \quad (6.1)
\]

where \( C_{pb} \) is the planktonic microbial concentration in suspension at time \( t \), \( D \) is the hydrodynamic dispersion coefficient, \( \lambda_{att} \) is the pseudo-first-order attachment rate coefficient, and \( u \) is the average bacterial velocity in the pore space (i.e. interstitial pore velocity). Note that, due to the phosphate free induction medium used in the column experiments, bacterial growth and decay were neglected in the model.

Equation 6.1 links the local rate of change in planktonic cell concentration to convection, dispersion, and attachment processes. Due to the colloidal nature of bacterial suspensions, classical colloid filtration theory (Yao et al., 1971) is the most commonly used approach in describing microbial transport and fate in porous structures (Yao et al., 1971; Harvery and Garabedian, 1991; Ryan and Elimelech, 1999; Schijven and Hassanizadeh, 2000). In CFT theory, first order kinetics, with a constant attachment rate coefficient, are used to describe spatial and temporal reduction of colloidal concentration in liquid suspension.
Thus, the packed-bed removal efficiency (i.e. fraction of the bacteria remaining in porous media) can be measured to evaluate the attachment rate coefficient.

In CFT, the attachment rate coefficient is linked to the single-collector removal efficiency ($\eta_r$) (O’Melia, 1985):

$$\lambda_{att} = \frac{a}{4} u \eta_r$$  \hspace{1cm} (6.2)

$$a = \frac{6(1-\varphi)}{d_c}$$  \hspace{1cm} (6.3)

where $a$ indicates wetted area in a packed bed of spherical collectors with an average diameter of $d_c$. In addition, in order to find $\eta_r$, the collision efficiency of a single collector ($\eta_c$) should be calculated. In the CFT, $\eta_c$ is a function of porous media geohydrological properties and indicates the probability that a suspended particle flows toward a collector and strikes that collector. Thus, $\eta_c$ reflects the efficiency of all possible transport processes and can be calculated using the following expression (Tufenkji and Elimelech, 2004):

$$\eta_c = 2.4 A_s^{1/3} N_R^{-0.081} N_{Pe}^{-0.715} N_{vdW}^{0.052} + 0.55 A_s N_R^{1.675} N_A^{0.125} + 0.22 N_R^{-0.24} N_G^{1.11} N_{vdW}^{0.053}$$  \hspace{1cm} (6.4)

where $A_s$, $N_R$, $N_{Pe}$, $N_{vdW}$, $N_A$, and $N_G$ are dimensionless numbers which are all described in Table 6-2 and the parameters used to calculate these numbers are summarized in Table 6-1. An equivalent spherical diameter of bacteria was required in these calculations.
Table 6-2: Dimensionless numbers used to calculate single collector collision efficiency.

<table>
<thead>
<tr>
<th>Dimensionless Number</th>
<th>Definition</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_R$</td>
<td>$\frac{d_{pb}}{d_c}$</td>
<td>Aspect ratio</td>
</tr>
<tr>
<td>$N_{Pe}$</td>
<td>$\frac{ud_c}{D_{SE}}$</td>
<td>Peclet number - ratio of convection to diffusion mass transfer</td>
</tr>
<tr>
<td>$D_{SE}$</td>
<td>$\frac{K_BT}{3\pi \mu_w d_{pb}}$</td>
<td>Stokes-Einstein diffusion - diffusion of spherical particles through liquid</td>
</tr>
<tr>
<td>$N_{vdW}$</td>
<td>$\frac{H}{K_BT}$</td>
<td>van der Waals number - ratio of van der Waals interactions to particles thermal energy</td>
</tr>
<tr>
<td>$N_G$</td>
<td>$\frac{d_{pb}^2(\rho_{pb} - \rho_w)g}{18\mu_w u}$</td>
<td>Gravity number - ratio of Stokes settling velocity to fluid average velocity</td>
</tr>
<tr>
<td>$N_A$</td>
<td>$\frac{H}{3\pi \mu_w u d_{pb}^2}$</td>
<td>Attraction number - particle deposition via interception</td>
</tr>
<tr>
<td>$A_S$</td>
<td>$\frac{2(1 - \nu^5)}{2 - 3\nu + 3\nu^3 - 2\nu^5}$</td>
<td>Porosity dependent parameter</td>
</tr>
</tbody>
</table>

Bacterial images, captured by phase-contrast microscopy (Axio Observer.A1, Zeiss, Germany), was analyzed by means of the AxioVision image processing and analysis system (AxioVs40©, Carl Zeiss MicroImaging) to find the equivalent spherical diameter of bacteria by determining area and dimensions of statistically meaningful numbers of bacteria (30 randomly selected bacteria in 5 different sets of samples) (Russ, 1995).
However, due to the repulsive colloidal interactions between colloids and collectors, not all the possible collisions actually result in attachment. Thus, in most aquatic systems, $\eta_r$ is lower than $\eta_c$ and can be calculated by:

$$\eta_r = \Omega \eta_c$$  \hspace{1cm} (6.5)

where $\Omega$ is sticking efficiency, indicating the probability of a striking particle irreversibly sticking to the collector. By neglecting dispersion, Equations 6.1, 6.2, and 6.3 can be combined and integrated to yield:

$$\Omega = -\frac{2}{3} \frac{d_c}{(1-\varphi)\eta_c L} \ln(RE_{pb})$$  \hspace{1cm} (6.6)

where $L$ is bed depth and $RE_{pb}$ represents bacterial recovery in column effluent (fraction of the bacteria entering the column that remained in the column effluent), described as:

$$RE_{pb} = \frac{\sum_{i=0}^{t} C_{pb}^i \Delta t_i}{C_{pb0} t}$$  \hspace{1cm} (6.7)

in which $C_{pb0}$ is initial bacterial concentration and $t$ indicates injection period of bacterial suspension. Therefore, by using Equations 6.2 to 6.7 and measuring the bacterial recovery in each experiment, bacterial attachment coefficient could be calculated. The obtained $\lambda_{att}$ value was applied to Equation 6.1 to simulate bacterial fate and transport in porous media. The model described above is a reactive flow process in porous media which was solved numerically by using the STARS™ simulation software (CMG, 2009). The domain was tessellated into a set of gridblocks and the governing equations were discretized by using a finite volume approach, and were solved on each gridblock (Bozorg et al., 2011). At initial time, the fluid is stationary and the concentration of cells
in the domain is equal to zero. Beyond \( t=0 \), fluid is injected through the injection port and withdrawn at the outlet. At the other boundaries, no flow conditions were imposed.

6.5 Results and Discussion

6.5.1 Parameter Estimation

Physical properties of packed beds are presented in Tables 6-1 and their calculated hydrodynamic characteristics are summarized in Table 6-3. The dispersivity was estimated for each column experiments by using the BTCs of conservative tracer (Figure 6-4) and used to simulate the corresponding experiment. Analyzing conservative tracer BTCs in each porous medium revealed that, except for the Packed-Bed 1, coating glass beads with biofilm had negligible impact on hydraulic properties. This implied that pore structures of media were not influenced significantly by formation of the thin biofilm layer (established during 24 hours) on glass beads. In addition, microscopic images of bacteria were analyzed using the image-processing software and an equivalent spherical diameter of 1.44 \( \mu \text{m} \) was calculated for HK44.

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Packed-Bed 1</th>
<th>Packed-Bed 2</th>
<th>Packed-Bed 3</th>
<th>Packed-Bed 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic Dispersion (D)</td>
<td>cm(^2)/s</td>
<td>3.66\times10(^{-3})</td>
<td>5.48\times10(^{-3})</td>
<td>1.10\times10(^{-2})</td>
<td>2.55\times10(^{-2})</td>
</tr>
<tr>
<td>Hydraulic Conductivity (K)</td>
<td>cm/s</td>
<td>6.46\times10(^{-3})</td>
<td>1.144\times10(^{-2})</td>
<td>9.74\times10(^{-2})</td>
<td>4.88\times10(^{-1})</td>
</tr>
</tbody>
</table>
Figure 6-4: Tracer breakthrough curves in 4 packed beds with different sizes of glass beads (Table 6-1). Similar results were obtained in the other trial.

6.5.2 Effect of Collector Size on Bacterial Removal Efficiency

In all the experiments conducted in clean packed beds, recovery of conservative tracer in the effluent was more than 90% after 3 pore volumes of solution had passed through the porous media. The non-recovered tracer could be due to losses during sampling and measurements. This signified that the columns were uniformly packed with no preferential flow paths or stagnation regions. However, in this set of experiments, bacterial removal efficiencies in columns were in the range of 48 to 13% (Table 6-4), increasing with reduced collector sizes, indicating that collector size had an impact on overall porous media removal efficiency. According to the CFT, diffusion, interception, and sedimentation are the three main processes that control packed bed removal.
Table 6-4: Bacterial concentrations in column effluents and determined attachment coefficients for each packed bed

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clean Glass Beads</th>
<th>Biofilm Coated Glass Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>$\eta_c$</td>
<td>1.1x10^{-2}</td>
<td>8.2x10^{-3}</td>
</tr>
<tr>
<td>RE_{pb}</td>
<td>0.52</td>
<td>0.64</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>0.17</td>
<td>0.27</td>
</tr>
<tr>
<td>$\lambda_{att}$ (1/s)</td>
<td>8.0x10^{-5}</td>
<td>4.9x10^{-5}</td>
</tr>
</tbody>
</table>

efficiency (Yao et al., 1971). Here, due to only a slight divergence in bacterial and water densities, sedimentation could be neglected. In the other two processes, removal efficiency was inversely related to the collector size and thus, reduced bacterial concentrations in effluent of columns with bigger collectors could be attributed in part, to the lower removal efficiency of coarse collectors. The obtained bacterial recoveries in the column effluents were applied to Equation 6.6 to calculate the sticking efficiency of the packed beds (Table 6-4). Subsequently, using the predetermined collision efficiencies ($\eta_c$), attachment coefficients were calculated (Equation 6.2) and applied to simulate transport processes in each column. According to the obtained results, predicted bacterial BTCs, combined with experimental results in Figure 6-5, indicated that the obtained attachment coefficients can be used to model bacterial transport and fate in the packed-beds. Studying BTCs of the conservative tracer in the packed-beds (Figure 6-4) showed a slow rise during the first 0.5 PV, highest increase at or near 1 PV, and a constant value after 2.5 PV, indicating full recovery of tracer (>90%) in column effluent. However, in all
Figure 6-5: Bacterial breakthrough curves combined with model prediction for bacterial transport in 4 packed beds with different sizes of glass beads (see Table 6-3 for details of each column). The general agreement between model predictions and experimental data indicates that the calculated sticking coefficients can be used to simulate bacterial migration in packed beds.

cases, bacterial concentration in column effluent declined and leveled off at a non-zero baseline concentration, resulting in a tailing effect with a slow rising bacterial concentration after 2.5 PV. This behavior can be explained by detachment of reversibly attached bacteria and our results show that this tailing effect persisted for at least 5 PV. Except for this slow rising tail in bacteria BTCs, BTCs of bacteria and conservative tracer were similar in shape, although the recovery of bacteria was always lower than the conservative tracer, which consistently exhibited more than 94% recovery.

During the breakthrough study of HK44, bioluminescence images were captured to visualize microbial transport and fate in porous media (Figure 6-6A). We have previously found that detected bioluminescence intensities (BI) can be correlated to the number of cells in porous media (Chapter 5). In addition, we showed that HK44 bacteria can keep
Figure 6-6: Bioluminescence images captured throughout the breakthrough experiments to visualize microbial transport and fate in the packed beds (see Figure 6-3 for specifications of each packed bed). Bacterial bioluminescence detected after eluting with 3 PV sterile induction medium represents the attached bacteria in the packed beds. (B) Model predictions for bacterial transport in columns with different sizes of glass beads using the calculated attachment coefficients. Good agreement between model predictions and experimental data indicates that the calculated sticking coefficients can be used to simulate bacterial transport behavior in packed beds.
their bioluminescence activity for at least 3 hours in the oxygen saturated induction medium with population density as high as $10^8$ cells/mL (Chapter 5 Section 5.5.1). Therefore, in the results presented here, a reduction in detected BIs could be correlated to lower numbers of cells present per unit volume of porous media. This fate and transport behavior of bacteria was confirmed by the model predictions, which was calibrated by calculated attachment coefficients for packed-bed (Figure 6-6B). According to these results, as the bacteria proceed through the porous media and depends on the porous media removal efficiency, portion of bacteria will attach to the porous matrix and thus, bacterial population density would decline during their migration. Bioluminescence images obtained after 3 PV confirmed that portion of bacteria were able to attach to the porous matrix and could not be washed out by 3 PV of eluting background flow. Higher number of cells was remained in porous media with smaller collector sizes indicating lower capability of coarse collectors in retaining bacterial cells. Modelling results were used to determine retained bacterial concentrations in packed-beds (Figure 6-6B).

6.5.3 Influence of Biofilm on Bacterial Attachment

Biofilm formation alters geohydrological properties of porous media which can interfere with bacterial transport and attachment. As biofilm forms on porous matrix, reduced permeability and porosity increase available surface area, $a$, (Equation 6.3) for bacterial deposition. In our study, except for the Packed-Bed 1 (Table 6-1) with 250 µm glass beads, practically similar hydraulic properties were obtained for the clean and biofilm coated porous media indicated that biofilm architecture and reduced bed porosity were not influencing factors in transport and fate of HK44 under the present experimental conditions. However, the presence of biofilm EPS may also change porous media
physicochemical surface characteristics, thereby altering electrostatic and van der Waals interactions between bacteria and porous media surfaces, as described by the DLVO theory (Elimelech et al., 1995; Wallker et al., 2004).

On the other hand, according to the extended DLVO theory, bacterial attachment to porous media solid surfaces would be enhanced by increasing surface hydrophobicity due to the reduction of connate water saturation (Elimelech et al., 1995). Bacterial EPS is normally composed of variable proportions of polysaccharides, lipopolysaccharides, lipids, proteins, and glycoproteins (Marshall 1991; Ellowood et al., 1979; Goodwin and Forster 1985; Horan and Eccles 1986; Grotenhuis et al., 1991; Jorand et al. 1995; Frolund et al., 1996). Therefore, it is expected that different polymers each contribute to bacterial attachment depending on different bacterial and substratum characteristics (Fletcher and Marshall, 1982; Paul and Jeffrey, 1985). Polymers which are able to form hydrogen bonding or strong electrostatic interactions, such as polysaccharides, may interact with hydrophilic surfaces (e.g. glass surface), whereas polymers with nonpolar sites, such as lipopolysaccharides, may be responsible for binding to hydrophobic surfaces (e.g. most of gram negative bacteria) (Abu-Lail and Camesano, 2003). Consequently, sticking efficiency of collectors can be significantly improved when are covered by EPS.

Figure 6-7A shows HK44 transport in biofilm coated porous media. Prior to the injection of bacterial suspension, each column was flushed by sterile induction medium, at the same flow rate of one PV per hour, until a negligible bacterial density was detected in the column effluent (<8x10^2 cells/mL). Bacterial bioluminescence detected in images captured at the initial stages of bacterial suspension injection into each column confirmed the presence of irreversibly attached bacteria (bacteria embedded in self-produced EPS)
Figure 6-7: Bioluminescence images captured to visualize microbial transport and fate in porous media coated with biofilm. Initial bioluminescence in captured in each experiment indicates presence of biofilm (i.e. bacteria embedded in EPS) in packed bead. Higher final bacterial bioluminescence detected after eluting with 3 PV sterile induction medium indicates bacterial attachment in packed beds (see Figure 6-3 for specifications of each packed bed). (B) Model predictions for bacterial transport in biofilm coated packed beds using the calculated attachment coefficients. Good agreement between model predictions and experimental data indicates that the calculated sticking coefficients can be used to simulate bacterial migration in biofilm coated packed beds.
in porous media. Based on the obtained bioluminescence images, comparatively higher BIs were detected in each column after eluting with 2 PV background flow. This suggested that higher numbers of cells were attached to the biofilm coated porous media compare to the clean packed beds. Modelling results were also used to determine retained bacterial concentrations in packed-beds (Figure 6-7B).

This observation was confirmed by studying bacterial BTCs when compared with BTCs of clean packed beds (Figure 6-8). Considering the BTCs, overall removal efficiencies in porous media were improved from 41% in Packed-Bed 1 to 91% in Packed-Bed 4. In Packed-Bed 1, due to the fine collectors and consequently, small pore sizes, formation of a thin biofilm layer on collectors impacted porous medium hydraulic properties, meaning that the calculated collision efficiency should be modified to account for biofilm thickness, available pore space, and interstitial pore velocity.

Therefore, other factors rather than collector sticking efficiency contribute to the observed enhanced attachment coefficient. For instance, polymeric bridging between EPS and bacteria may play an important role in removal efficiency of low permeability Packed-Bed 1 (Figure 6-2). However, in the other 3 porous media, due to the identical hydraulic properties confirmed by conservative BTCs, lower bacterial concentrations in column effluent could be related to higher sticking efficiency in porous media. In Packed-Bed 4 with no variation in hydraulic properties, 91% improvement in removal efficiencies (Table 6-3) in presence of biofilm, indicates that higher portion of the bacteria collided with a collector will adhere to it due to its higher sticking efficiency.
Figure 6-8: Bacterial breakthrough curves combined with model prediction for bacterial transport in 4 packed beds with different sizes of glass beads coated with EPS. The general agreement between model predictions and experimental data indicates that the calculated sticking coefficients can be used to simulate bacterial migration in EPS coated packed beds.

6.6 Conclusions

In order to better understand microbial processes in porous media, bacterial fate and transport was studied and the effect of bacterial self produced extracellular polymeric substances on their adhesion to porous matrix was investigated in porous media with different hydraulic properties. A genetically engineered bioluminescent bacterium was used to visualize bacterial transport, fate, and retention in translucent porous media. Bacterial breakthrough experiments revealed that formation of a thin biofilm layer (i.e. bacteria embedded in self produced EPS) enhances bacterial attachment. Using the extended DLVO theory, it was concluded that covering porous matrix with a thin layer of EPS can significantly change hydrophobicity of solid surfaces and thereby, modifies the
porous media sticking efficiency. Using the colloidal filtration theory, observed results were used to calculate sticking efficiencies in clean and biofilm coated porous media and subsequently, calculated values were used to simulate bacterial transport in each medium. This work can help to better understand and predict bacterial transport behaviors in porous structures in situations in which initial bacterial attachment and EPS secretion alter physicochemical, and thus, porous medium sticking efficiency.
Chapter 7

Effects of Porous Medium Hydraulic Properties on Biofilm Growth Patterns in Porous Media

7.1 Preface

The present thesis is based on refereed papers that have already been published or are in the process of publication. Unavoidably, there is some repetition between this chapter and Chapters 4 and 5, particularly in the Introduction section where biofilm and its applications are introduced, as well as in the Materials and Methods, where experimental procedures are described. Therefore, in this chapter, sections of Introduction and Materials and Methods may be safely skipped without loss of coherence. In addition, more comprehensive details of experimental procedures used to prepare culture media, sample the porous media, prepare biofilm samples, and determine bacterial population, as well as the assays used to measure bacterial DNA and protein concentration are provided in Appendices A through G. Also, MATLAB codes used to process the bioluminescence images are summarized in Appendix J.
7.2 Abstract

Biofilms are surface attached communities of microorganisms embedded in a self-produced organic matrix of extracellular polymeric substances (EPS). Porous media, which inherently possess high specific surface areas, can be promptly colonized by planktonic bacteria which establish biofilm on solid matrix. Biofilm development in porous structures can gradually lower the available void space for fluid flow making it increasingly difficult for fluids to flow through the pore network. This process, known as bioclogging, can significantly influence the effectiveness of processes that rely on fluid flow through porous media. In such processes, spatiotemporal development of biofilm is one of the major factors determining process performance. To study biofilm evolution in porous media and better understand the parameters affecting biofilm spatial expansion, biofilm growth was studied in porous media with different hydraulic properties. By using *Pseudomonas fluorescens* HK44, an engineered bioluminescent bacterial strain, biofilm growth patterns in a two-dimensional flow field under constant pressure gradients were monitored. Results revealed that biofilms grow predominantly in upstream regions against pressure gradient and toward the nutrient injection port in porous media with fine pore sizes and low permeabilities, whereas in porous media with coarse pore sizes and elevated permeabilities, biofilm primarily dispersed toward the downstream regions. The observed biofilm growth patterns were linked to bioclogging of regions within the porous media saturated with biofilm as well as the pore size distribution in porous media.
7.3 Introduction

In nature, bacteria can be found in abundance in almost any aqueous environment. Typically, to protect themselves against harsh environmental conditions and harmful factors, bacteria tend to be sessile (i.e. surface attached) in fully hydrated and structured communities referred to as biofilms (Characklis et al., 1990; Costerton, 1995). Biofilms consist of an accumulation of bacteria attached to a substratum encapsulated by self-produced extracellular polymeric substances (EPS). Biofilms are ubiquitous in almost any moist system and it has been estimated that over 90% of all bacteria live as surface attached bacteria in fully structured communities (Characklis et al., 1990). Accordingly, a wide variety of medical, environmental, and industrial applications, such as food processing (Pereira and Vieira, 2001; Dogan and Boor, 2003), marine vessels (Townsin, 2003), water and wastewater treatment (Waybrant et al., 1998; Cunningham et al., 2003; Golab et al., 2006; Davis et al., 2007), bioremediation of contaminated soil (Cunningham et al., 1997; Nyman et al., 2002; Cunningham et al., 2003; Komlos et al., 2004), petroleum recovery (Cusack et al., 1992; Lappin-Scott et al., 1988; MacLeod et al., 1988), and water distribution systems (LeChevallier, 1990; Camper, 1993) can be subject to biofilm growth.

In porous structures, the high specific surface area (surface area to volume ratios) facilitates bacterial attachment and colonization, thereby making biofilm the most dominant form of bacterial growth. As a consequence of biofilm development, the hydrodynamic properties of porous media can change over time through a process known as bioclogging (Baveye et al., 1998). When an aqueous phase containing dissolved nutrients that support microbial growth passes through a porous medium, microbial cell
proliferation, combined with EPS secretion by microorganisms, results in biofilm accumulation in the pore spaces (Baveye et al., 1998; Characklis et al., 1983; Cunningham et al., 1991; Taylor and Jaffe, 1990d). As biomass and EPS accumulate in the pore space, flow becomes restricted, limiting mass transport of nutrients to the cells (Taylor and Jaffe, 1990d; Vandevivere and Baveye, 1992a,b). This means that the geohydrological properties, biofilm saturation (i.e. fraction of void space occupied by biofilm), bacterial activity, and bacterial population dynamics are strongly related processes. Such coupled interaction between biofilm accumulation and hydraulic properties of porous media has been shown to control or significantly impact the performance of biofilm-related processes.

Bioclogging is most often associated with detrimental consequences (Baveye et al., 1998). For instance, bioclogging can reduce the performance of sand filters used in wastewater treatment (Gray, 1981) or drinking water (Urfer et al., 1997). Studies have demonstrated that bioclogging can adversely influence the success of bioremediation in aquifers (Thullner et al., 2002; Cunningham et al., 2003; Seifert and Engesgaard, 2007). Also, during groundwater discharge, bioclogging of the discharged wells and nearby aquifers can also result in operational problems (van Beek and van der Kooij, 1982). Similarly, injection of water which may contain nutrients into an oil bearing formation to enhance oil recovery can result in microbially mediated formation damage (i.e. biofouling) in the vicinity of the injection site (Cerini et al., 1946; Clementz et al., 1982; Cunningham et al., 1991).
However, the ability to engineer biofilms and control their growth and activity in porous media can provide significant opportunities to improve the performance of industrial and environmental processes influenced by biofilm formation and those that use biofilms to achieve a process goal. For example, development of permeable, impermeable, and semipermeable biobarriers has been proposed for the control and remediation of contaminated soil and groundwater (Cunningham et al., 1997; Waybrant et al., 1998; Benner et al., 1999; Hiebert et al., 2001; Nyman et al., 2002; Cunningham et al., 2003; Komlos et al., 2004) (Figure 7-1). Reactive biobarriers can be established to enhance active biodegradation of contaminants. Also, engineering biofilms and stimulation of

![Figure 7-1: Biofilm can be engineered to form reactive permeable biobarriers to increase residence time and remediate subsurface contaminants. Also, impermeable biobarriers can be developed to prevent migration of contaminants to preserve groundwater resources.](image)
microbial growth at specific subsurface locations is the basis of in situ formation of biobarriers to control subsurface flow as a means of impeding and controlling the transport of pollutants to groundwater resources (Sharp et al., 1999; Mitchell et al., 2008; Cunningham et al., 2009). Such barriers are engineered to provide maximum bioclogging (i.e. reduction of permeability) by stimulating formation of thick biofilm to either reduce the groundwater flow through certain regions of the subsurface, or direct the flowing aqueous phase toward a certain direction (e.g., location of treatment plant) (Figure 7-1). In addition, as illustrated in Figure 7-2, premeditated stimulation of microbial growth has also been used to bioclog highly permeable fractures in reservoirs (Ross and Bickerton, 2002), thereby reducing water flow through preferential flow paths (i.e. thief zones) and enabling enhanced oil production during secondary oil recovery (Cusack et al., 1992; Lappin-Scott et al., 1988; MacLeod et al., 1988). Severe bioclogging is usually achieved

Figure 7-2: Application of bioclogging in microbial enhanced oil recovery. Biofilm growth and development of impermeable biobarriers enhances the amount of recovered oil by bioclogging the preferential flow paths with high permeability. As a result, the injected water will go through the less permeable regions and sweep the trapped oils and push them through the production wells.
by promoting the production of copious amounts of EPS by indigenous cells or through bioaugmentation with organisms known to have such capabilities.

However, in order to engineer biofilms and establish such biobarriers at specific regions within the porous media, influences of key environmental (e.g. porous media hydraulic properties) and operational (e.g. fluid flowrates and nutrient concentrations) factors affecting structure and spatial development of biofilms in porous media should be well understood. A number of studies have investigated biofilm growth and bioclogging in porous media. In biofilm studies, bacteria are often considered to be colloids transported by aqueous phase within the porous media (Katsikogianni and Missirlis, 2004) and thus, the spread of such colloidal particles in porous media has been simulated primarily by passive transport via fluid convection.

However, experimental studies have revealed that such a simple transport mechanism (i.e. passive convection) cannot explain the observed evolution of biofilm in porous structures. Diverse biofilm growth patterns have been observed in different experimental studies conducted in porous media with different characteristics. For example, biofilm growth toward the nutrient injection port and against the pressure gradient has been reported in mesoscale laboratory experiments (Kildsgaard and Engesgaard, 2001; Seki et al., 2006; Bozorg et al., 2011). Also, in different column experiments (Taylor and Jaf fê, 1990c; deLeo and Baveye, 1997; West et al., 2007), it has been reported that flow reduction during biofilm growth in porous medium was a result of biofilm development in the upstream sections of the column and near the chamber inlet rather than the downstream regions. Additionally, bacterial distribution profiles in such experiments
revealed maximum biomass at the position closest to the inlet compared to a measured low cell concentrations at the vicinity of the chamber outlet. In an experiment conducted by Bozorg and his colleagues (2011), although biofilm growth was observed primarily toward the inlet port, biofilm dispersion with lower intensity was simultaneously observed at downstream regions. However, although biofilm dispersion in regions downstream of the inoculation zones was explained by passive convection of detached cells via the background flow, little or no explanations has been provided in such experiments to justify biofilm development against the pressure gradient at upstream regions. Therefore, processes affecting such biofilm behavior are not clarified.

Considering the significant impact that biofilms can have on porous media based applications, it is important to gain better understanding on how different parameters affect biofilm spatial development in porous media. More comprehensive knowledge regarding biofilm behavior within pore networks would aid the implementation and establishment of engineered biofilms in porous structures. Here, by using a bioluminescent bacterium, biofilm spatial expansion was studied in porous media with different hydraulic properties. Development of biofilm was monitored in translucent porous media and the observed variations in biofilm growth patterns could be explained by the specific geohydrological properties of each medium.
7.4 Materials and Methods

7.4.1 Microorganism

The bioluminescent reporter strain *Pseudomonas fluorescens* HK44 (hereafter referred to as simply HK44), containing the stable plasmid pUTK21, was provided by the University of Tennessee, Center for Environmental Technology (Knoxville, Tennessee). The plasmid was made by the insertion of the *lux* CDABE gene cassette of the marine bacterium, *Vibrio fischeri*, into the upper naphthalene degradative pathway of catabolic plasmid Nah7. Therefore, the *lux* genes can be induced by naphthalene, or by salicylate, which is a metabolite of naphthalene degradation (King et al., 1990). In this study, due to the high solubility of salicylate salts, salicylate was used to induce the *lux* gene of HK44. The plasmid encodes tetracycline resistance so all growth media contained 15 mg/L tetracycline to prevent plasmid loss.

7.4.2 Growth and Induction Media

To preserve HK44 planktonic phenotype, stock cultures of HK44 were stored in 25% glycerol at -80° C. From the frozen stocks, cells were retrieved at 27° C on Luria broth agar (LB agar) plates which contained 5 g NaCl, 10 g tryptone, 5 g yeast extract, and 15 g agar per one liter distilled water (pH 7.0) and used to prepare experimental inoculum.

Inocula were generated in an oxygen-saturated, nitrate-free growth medium, that consisted of glucose (1 g/L) as the main carbon source and the mineral salts medium (MSM) composed of: MgSO₄, 0.4 g/L; CaCl₂·2H₂O, 0.1 g/L; NH₄Cl, 0.4 g/L, NaCl, 8 g/L; KCl, 0.2 g/L; NaH₂PO₄, 1.15 g/L; K₂HPO₄, 0.26 g/L; HCl, 0.00366 g/L; FeSO₄·7H₂O, 0.021 g/L; H₃BO₃, 0.0003 g/L; MnCl₂·4H₂O, 0.001 g/L; CoCl₂·6H₂O,
0.0019 g/L; NiCl$_2$.6H$_2$O, 0.00024 g/L; CuCl$_2$.2H$_2$O, 0.00002 g/L; Na$_2$EDTA.2H$_2$O, 0.01 g/L; ZnSO$_4$.7H$_2$O, 0.00144 g/L; Na$_2$MoO$_4$.2H$_2$O, 0.00036 g/L. The pH of the growth medium was adjusted to 7.0 using 1 M NaOH.

Additionally, induction medium was prepared to induce the HK44 lux gene to luminesce by adding 0.1 g/L (final concentration) of salicylate to the growth medium. It has been reported that the removal of all phosphate sources restricts HK44 growth while keeping the other metabolic pathways (including those involved in bioluminescence) intact (Webb et al., 1997; Oates et al., 2005, Bozorg et al., 2011). Therefore, to dedicate nutrient sources to bioluminescence during the induction phase, all phosphate sources were removed from the induction medium.

As the pUTK21 plasmid used in HK44 encodes tetracycline resistance marker, growth and induction media were also supplemented with 30 mg/L (final concentration) tetracycline to prevent plasmid loss. Tetracycline solution (EMD Chemicals, OmniPur® EM-8990) was prepared in 50% ethanol solution (v/v of water) and added via a 0.2 μm filter to autoclaved media. In addition, our previous studies on HK44 bioluminescence indicated that saturating the medium with pure oxygen significantly increases the constant bioluminescence period while keeping the maximum bioluminescence value intact. Hence, to reduce the negative impact that oxygen availability can have on bacterial bioluminescence (Bozorg et al. 2011), prior to injection, all media were saturated with pure oxygen (Praxair Inc.) (please see Appendix A for more details).
7.4.3 Inoculum Preparation

Following overnight incubation of the retrieved bacteria in LB medium, 0.1 mL of the culture (OD\textsubscript{550}=0.2) was inoculated into 30 mL batch culture of fresh growth medium. After inoculation for 24 hours on a rotary shaker (Heidolph Unimax 2010, Germany) at 150 rpm and room temperature, cells were harvested by centrifugation (Beckman Coulter\textsuperscript{®}, X-22R) at 5000 rcf for 20 min, washed in a phosphate buffered saline (PBS) solution consisting of NaCl (24 g/L), KCl (0.6 g/L), NaH\textsubscript{2}PO\textsubscript{4} (3.45 g/L), K\textsubscript{2}HPO\textsubscript{4} (0.78 g/L), centrifuged again and resuspended to 6x10\textsuperscript{6} cells/mL in growth medium supplemented with salicylate (0.1 g L\textsuperscript{-1}) to induce bioluminescence. After confirming that bioluminescence could be visually observed, this culture was used as inoculum in flow chamber experiments. Bacterial population densities were determined by measuring light absorbance at 550 nm (Appendix E) using a spectrophotometer (DU 730, UV/Vis Spectrophotometer, Beckman Coulter\textsuperscript{®}).

7.4.4 Porous Media

To evaluate the effect of grain size on biofilm growth in porous media, three different sized translucent acid-washed glass beads (Sigma–Aldrich, G1277, G8772, and Z273627), with effective bead diameters of 0.25, 0.5, and 2 mm were used as porous media. Porosities of the packed beds were determined gravimetrically by using the density of glass beads (\(\rho_g\)) and the measured bulk density of each porous media (\(\rho_{bulk}\)) (Fetter, 1994):

\[
\varphi = 1 - \frac{\rho_{bulk}}{\rho_g} 
\]  

(7.1)
where, $\varphi$ is the porosity of the porous medium. In addition, as indicated by Glover and Walker (2009), effective pore diameter decreases with porosity and increases with grain size as following:

$$d_g = \sqrt[3]{\frac{\alpha \theta^2}{8 \varphi^2}} d_p$$  \hspace{1cm} (7.2)

where $d_g$ is the effective grain diameter, $d_p$ is effective pore diameter, $\alpha$ is adjusting parameter, and $\theta$ is cementation exponent. For spherical grains, $\theta=1.5$ and $\alpha=8/3$ (Bernabé and Revil, 1995; Glover et al., 2006), and thus, Equation 7.2 becomes:

$$d_g = \sqrt[3]{\frac{2.25}{3\varphi^3}} d_p$$  \hspace{1cm} (7.3)

Pertinent characteristics of porous media are summarized in Table 7-1.

<p>| Table 7-1: Characteristics of 3 different porous media used to study biofilm spatiotemporal development. |
|---|---|---|---|</p>
<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Porous Medium</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Bead Size ($d_b$)*</td>
<td>mm</td>
<td>0.25±0.044</td>
<td>0.5±0.087</td>
</tr>
<tr>
<td>Sieve Size</td>
<td>-</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td>Porosity ($\varphi$)</td>
<td>-</td>
<td>0.44</td>
<td>0.39</td>
</tr>
<tr>
<td>Pore Size ($d_p$)</td>
<td>mm</td>
<td>0.084</td>
<td>0.141</td>
</tr>
<tr>
<td>Permeability (K)</td>
<td>cm$^2$</td>
<td>3.78x10$^{-8}$</td>
<td>7.92x10$^{-8}$</td>
</tr>
</tbody>
</table>

* Provided by manufacturer
Although the glass beads were prewashed by the manufacturer, prior to use, the glass beads were washed twice with distilled water to remove fines and autoclaved at 121°C for 20 minutes.

7.4.5 Flow Chamber

Figure 7-3 illustrates the flow chamber used to perform biofilm experiments in a two-dimensional flow field. This flow chamber was custom designed by the candidate to facilitate evaluation of permeability throughout the flow chamber (achieved by determining pressure heads) while simultaneously monitoring biofilm evolution in porous media. The chamber was consisted of two main parts; a base to hold the glass beads, and a glass cover. The chamber base was made of a 41 cm long by 23 cm wide by 5 cm thick Plexiglas, with a 25 cm by 14 cm by 1 cm hole at the middle which had been removed to house porous media (Figure 7-4). Therefore, since the thickness of the porous media in flow chamber is much smaller than its length and width, flow chamber simulated a two dimensional flow field. The bottom of the Plexiglas chamber base was drilled with a series of holes to fit ports be used for pressure measurements all over the porous media (Figure 7-4). The ports were sealed with high-temperature silicon sealer to allow injection or sampling via syringe and thus, could also be used for inoculation, sampling, and injection of solutes (e.g. tracer solution) at any point within a porous medium. In addition, to prevent formation of preferential flow paths due to injection via the inlet ports, the flow chamber was equipped with two inlet and outlet sockets (Figure 7-3 and 7-4) each separated from the porous medium by a perforated plate to inject and collect the growth medium homogeneously into and from the porous media.
The void space in the flow chamber was outfitted with 0.1 mm steel mesh and filled with different sizes of glass beads (Table 7-1). A fluorocarbon rubber O-ring (Viton®, Dupont Dow Elastomers) was used as a seal between the cover glass and the chamber base by using C-clamps. To enable complete sterilization, all chamber materials were selected to withstand autoclaving.

![Figure 7-3: Custom designed flow chamber used to perform biofilm experiments under two-dimensional flow field in porous media. The flow chamber consisted of two main pieces; a base and a glass cover. The base was made of Plexiglas with a hole at the middle which had been removed to house porous media. The bottom of the Plexiglas base was drilled to fit ports for pressure evaluation all over the porous media. The ports are sealed with high-temperature silicon sealer to allow injection or sampling via syringe. Also, these ports could be used for inoculation and injection of solutes at any point within the porous media. Fluorocarbon rubber O-ring (Viton®, Dupont Dow Elastomers) was used to seal the cover glass to the base by using C-clamps.](image-url)
Figure 7-4: Schematic of the base of the flow chamber used in flow experiments to study biofilm evolution in porous media. The chamber base was made of 41 cm long, 23 cm wide, and 5 cm thick Plexiglas. A 25 cm by 14 cm by 1 cm hole was cut out of the base that was filled with glass beads to provide two-dimensional flow field to investigate biofilm behavior under flow conditions. The void space was outfitted with perforated plates, covered by steel mesh, to keep porous media in place. In addition, side holes were drilled at the side of the base to facilitate packing the flow chamber with glass beads. The chamber was also equipped with two inlet and outlet sockets to provide uniform fluid flow with minimal preferential flow paths within the porous media.
7.4.6 Chamber Sterilization

Preliminary attempts to autoclave the chamber assembly after packing with glass beads were not successful, due to the bubble formation within the porous media which could not be removed later by flushing the media. Therefore, to minimize bubble formation in porous media, after assembly, the empty chamber was fully filled with deionized water and its inlet and outlet ports where blocked. Subsequently, the chamber was autoclaved at 121°C for 20 minutes and allowed to cool down prior to being packed with sterile glass beads. All the fittings, connectors, and tubing were also autoclaved separately and were assembled within a laminar flow biological safety cabinet (Forma Class II Thermo Electron Corporation). Subsequently, autoclaved flow chamber was packed with sterile glass beads in the laminar flow biological safety cabinet. Using a sterile funnel, glass beads were added to the flow chamber from designated ports at the side of the chamber (Figure 7-4) whereas one of the other side ports was kept open to take out the overburden water. After packing, fully saturated porous media as well as all the surfaces were exposed overnight to UV light (254 nm) to ensure complete sterilization. Subsequently, while oriented in vertical position, several pore volumes of the autoclaved growth medium were pumped through the system (Gilson Minipuls 3) to saturate the porous media with growth medium. The growth culture (as well as all the other solutions) was stored in autoclaveable storage bottles (VWR®) with ventilated polypropylene closures equipped with 0.2 µm filters. In addition, to avoid contamination of tubes and inlet/outlet ports, three way valves were used at each connection port to facilitate sterilization with alcohol as needed.
7.4.7 Experimental Procedures

7.4.7.1 Operation of Flow Chamber

Each experiment was initiated by inoculation of visibly bioluminescent cells (please see Section 7.3.3) into the saturated porous media via the inoculation port on the bottom of the Plexiglas chamber base (Figure 7-4). In each experiment, injection of inoculum lasted inoculum was injected to the porous medium to inoculate a bulk volume of approximately 1.8 cm³ (i.e. inoculated region with a circular surface area of 1.5 cm in diameter). Brilliant Blue FCF dye (ACROS Organics) was added to the inocula to enhance visibility of inoculated regions. Results from the preliminary experiments conducted on planktonic cells confirmed that the Brilliant Blue FCF dye did not have any negative effects on HK44 growth and bioluminescence activity.

After inoculation, to prevent bacterial washout, continuous background flow of MSM was not initiated until 18 hours to allow a stable layer of attached bacteria be established in inoculated region. Different time intervals, ranging from one hour to 5 days, have been used in experimental studies to provide sufficient time for bacterial cells to attach to the porous matrix (Bozorg et al., 2012; Clement et al., 1997; Cunningham et al., 1991; Engesgaard et al., 2006; Kildsgaard and Engesgaard, 2001; Vandevivere and Baveye, 1992a). In the present study, it was concluded that 18 hours attachment period was enough to prevent bacterial washout from inoculated regions. Following initial bacterial attachment, glucose solution (1 g/L) was injected through the nutrient injection port (Figure 7-4), and the MSM was supplied to the background inlet ports via Mariotte tubes (SMS®, Arizona, USA), both kept at the same pressure head. The inoculated chamber was maintained at a room temperature of 21°C ± 1°C during the course of the biofilm
experiment. The constant pressure gradient between chamber inflow and outflow applied by the Mariotte tubes enabled the porous media permeability to be evaluated during the biofilm experiments, thereby, facilitating the investigation of bioclogging in porous media. Also, in order to visualize the flow path of glucose solution (i.e. main carbon source) in porous media containing biofilm, tracer experiments were performed by adding Brilliant Blue FCF dye to the glucose solution and its distribution was monitored by taking photos from above of the flow chamber. Flow rates of the injected fluids were monitored by using calibrated in-line flowmeters (BEL-ART Riteflow®) at both the inlet and outlet of the chamber. Discharge rates were measured to calculate average fluxes in the flow chambers. When measuring discharge fluxes, injection of glucose solution from the injection port was stopped and MSM was supplied to the inlet ports only. Pressure distribution was also monitored throughout the porous media by using highly accurate pressure transducers with micro-machined silicon sensor (PX409-USB, Omegadyne Inc.). Together with the discharge rates, these pressure measurements enabled calculation of permeabilities throughout the porous medium. The biofilm experiments were finished after 12 Days of continuous flow operation in each porous flow chamber. All experiments were conducted in duplicate.

7.4.7.2 Bioluminescence Imaging

The imaging system and image processing approaches have been described in detail in chapters 4 and 5, and so will only be described here briefly. To capture bioluminescence images, all flow chamber experiments were conducted in a dark box (Figure 7-5) to minimize interference between bacterial bioluminescence and other light sources. To induce bacterial bioluminescence in flow chambers, once a day, the fluid being fed
Figure 7-5: Dark box used in the experiments to capture bioluminescence images. Once the door was placed on the front of the box, it provided a completely dark environment to ensure minimal interference of bacterial bioluminescence by other light sources.
through the injection port was switched from glucose solution to induction medium, whereas the background flow of MSM was kept unchanged. After the induction medium passed through the developed biofilm in porous media, a bioluminescence image was taken using a 14-bit digital CCD camera (Progres MFcool CCD camera, Jenoptik, Germany) with an interline 2/3" Sony ICX285AL 1.4 megapixel progressive scan monochrome CCD sensor encased by a hermetically sealed, nitrogen-flushed capsule cooled by a Peltier element, a heat sink and a fan during long exposures. This camera was equipped with a Computar Megapixel lens with a 35 mm focal length and an f1/4 focal ratio which was protected by a longpass UV filter (Edmund Optics, 25.5 mm) to permit passage of all fluorescent light. The camera was controlled by the CapturePro software (ProgRes® CapturePro 2.7.7, Jenoptik, Germany).

In all the experiments, a 5 min exposure time with full aperture (f1/4) was used. To obtain comparative bioluminescence images over time, it was necessary to maintain the chamber in a fixed position relative to the camera and keep the exposure time unvarying in all experiments. Therefore, the camera was placed 110 cm above the flow chamber to image the whole porous medium in one image. Consistent imaging procedures made it possible to compare different bioluminescence images, and therefore, evaluate the impact of porous media characteristics on biofilm evolution in porous media. Facilitated by the CapturePro software, a background image was taken prior to induction and then subtracted from each bioluminescence image recorded after induction to ensure minimal interference of surrounding optical noise.
7.4.7.3 **Bacterial Cell Enumeration**

To determine bacterial cell distribution in porous media, at the conclusion of each experiment, porous medium was collected and removed from sampling regions at upstream and downstream zones (Figure 7-4) by using a 7 cm diameter hollow tube. Each porous medium sample was mixed well and then aliquoted into separate test tubes containing a known volume of PBS and vortexed and sonicated repeatedly to detach biofilm from glass beads (Heersink 2003; Bozorg et al., 2012) (Appendix C). Bacterial cell number in the obtained liquid aliquots was then determined by measuring the mass of total DNA based on the selective binding of DNA to a silica-based membrane. Cells membrane proteins were degraded by Proteinase K and chaotropic salt and their encapsulated DNA was extracted from the lysed cells, providing an opportunity for the extracted DNA in the chaotropic salt to stick to the silica-based membrane (Qubit® dsDNA Assay Kit, Invitrogen, Oregon, USA). Additionally, to minimize RNA contribution in measurements, RNase A was added to the lysate to degrade RNA and purify DNA samples (please see Appendix F for more details).

Since a single cell strain was used, the total DNA weight could be used to determine number of bacterial cells. By considering the mass of the PUTK21 plasmid added to HK44 and the published mass of DNA in wild type *Pseudomonas fluorescens*, the total mass of DNA per cell was calculated to be equal to $7.843 \times 10^{-15}$ g (King 1990), which was consistent with the value obtained experimentally. A standard curve, correlating the measured mass of DNA to the number of cells was generated by manual cell counting in a counting chamber (Hemacytometer Set, Hauser Scientific) (Appendix G) and used to estimate number of cells based on the measured mass of DNA.
7.4.7.4 Protein Measurement

To evaluate amount of EPS at downstream and upstream regions, prepared porous media samples were used to assess total protein concentration (i.e. combination of EPS and intracellular proteins) by using the bicinchoninic acid (BCA) method (Smith et al., 1985) (Appendix H). Bacterial protein extraction lysis buffer (Bacterial–PELB, GBiosiences®) was used to extract intracellular proteins. Absorbance of light (wavelength 562 nm) against the fresh reagent was measured spectrophotometrically and compared to a standard curve (Appendix H) generated by using bovine serum albumin (BSA). Also, in order to differentiate between EPS and encapsulated bacterial proteins, porous media samples were also used to evaluate intracellular protein content. As described for cell enumeration (Section 7.4.7.3), biofilms were detached from the glass beads and bacteria were harvested from each sample by centrifugation (5000 rcf, 4°C for 20 minutes) and washed twice with PBS. Total intracellular protein was then measured by using the same method described above.
7.5 Results and Discussion

7.5.1 Hydraulic Variations

Fluid fluxes through the porous media were monitored throughout each experiment. Nonetheless, gradual flux reduction was observed in all three porous media all along biofilm development. As shown in Figure 7-6, over the course of 12 days experiments, the greatest flux reduction of 59.0% was detected in porous medium A which had the finest grain size (23.1 and 4.2% flux reductions were recorded for porous media B and C, respectively). Considering the applied constant pressure gradient, reductions in fluid fluxes can be explained by the reduction of average permeability caused by biofilm development in porous media, or simply bioclogging. Evolving biofilms progressively occupy greater proportions of the void space, thereby lowering porous media permeability and restricting fluid transport.

![Figure 7-6: Flux changes during biofilm experiments in 3 different porous media (please see Table 7-1 for details of each porous medium). Values are means ± standard deviations (error bars).](image-url)
Figure 7-7 shows hydraulic head profiles along the flow chamber for each porous medium. At each distance from the inlet of the porous medium, 4 or 5 pressure heads were measured via the pressure ports at the bottom of the flow chamber base (Figure 7-4) and averaged to determine overall hydraulic head at each level. At the beginning of the experiments, hydraulic head profiles revealed constant gradients along all three flow chambers, indicating consistent flow across the porous media. However, toward the end of the experiments, a variety of profiles were observed. In porous medium C, hydraulic head profile remained unchanged during the experiment, meaning that the biofilm growth in that porous medium was not able to significantly impact hydraulic properties and thus, fluid transport was relatively unaffected during biofilm growth over 12 days in porous medium C. Conversely, evolving hydraulic head profiles in porous media A and B revealed steeper gradients at the vicinity of the inoculation regions which could be related to intensive bioflocculation at such regions. Due to the small pore sizes and accordingly, low permeabilities of porous media A and B (Table 7-1), compared to porous medium C, biofilm growth could have more severe impacts on permeability in such porous structures.
Figure 7-7: Hydraulic pressure gradient across the flow chamber determined by averaging pressure readings at each distance from the inlet of the porous medium, facilitated by pressure ports at the bottom of the flow chamber base (please refer to Figure 7-4 for location of pressure ports at each level).
By using the average fluxes and the measured hydraulic heads throughout the porous media, overall permeabilities, as well as local permeabilities upstream and downstream of the inoculation zone (6.5 to 16.5 cm and 16.5 to 25 cm from the inlet of the porous medium as shown in Figure 7-4) were calculated (Figure 7-8). Based on the obtained results, permeabilities of upstream and downstream regions in porous media A and B revealed different patterns when compared with porous medium C. Higher permeability reductions were observed in upstream regions of porous media A and B, whereas in porous medium C, permeability of upstream zones was practically intact all over the experiment. This implied that in porous media C, biofilm spread mainly in downstream regions while in porous media A and B, biofilm primarily developed at the upstream of the initially inoculated zones.

Figure 7-8: Comparison between overall relative permeability (compared to day 1) and relative permeability of upstream and downstream regions in 3 porous media A, B, and C (please check Table 7-1 for details of each medium). Values are means ± standard deviations (error bars).
7.5.2 Spatial Development of Biofilm

To study effect of porous medium characteristics, specifically pore size distribution, on biofilm spatial expansion, biofilm development was monitored over 12 days in porous media with different bead sizes. Figure 7-9 illustrates dynamic biofilm growth patterns in translucent porous media A, B, and C as monitored by daily imaging of the induced cells in flow chambers. Following inoculation of the porous media and prior to start of the background flow, the Day 1 images were taken to show the initially colonized regions in the flow chamber. Bacterial bioluminescence detected in Day 1 images confirmed attachment of bacterial cells in the inoculated regions.

In porous medium A, biofilm spread from the inoculated region (1.5 cm diameter) on Day 1 to a region of more than 8.6 cm in width by day 12. Starting on Day 5, bacterial bioluminescence revealed a horseshoe-like spatial pattern with a dark region at the vicinity of inoculation zone. As shown in Figure 7-9, this pattern remained qualitatively the same and expanded over time toward the injection port. Interestingly, the dark interior regions of the colonized zone had earlier emitted detectable bioluminescence. For instance, regions peripheral to the inoculation port changed from being highly luminescent on Day 5 to less so on subsequent days. This observation indicated that although HK44 existed in such regions (confirmed by destructive sampling at the conclusion of experiment), they did not respond to the application of induction medium. This could be related, in part, to bioclogging of these regions or the upstream sections which restricted availability of induction medium to HK44. Similar growth pattern was observed in porous medium B but with higher dispersion at downstream zones and lower expansion upstream of the inoculation zone toward the injection port.
On the other hand, biofilm growth in porous media C was different than the other two media and no dark region detected in bioluminescence images. Also, in porous medium C, biofilm was dispersed primarily in downstream regions. Such diverse biofilm evolutions can be linked, in part, to permeability and pore size distribution of porous media. Compared to the other two media, moderately large pore sizes in porous media C led to different biofilm growth patterns.
medium C can facilitate biofilm growth to higher extent on glass beads, which in turn promotes bacterial detachment from the established thick biofilm. Detached bacteria can be then carried by the fluid flow and reattach to the glass beads or be trapped in pore throats and initiate biofilm formation at downstream regions. Therefore, it is more likely that in porous medium with large pore sizes, such as porous medium C, biofilm mainly develops around the inoculation zone and spreads via passive convection at downstream regions.

7.5.3 Biological Analyses

Qualitative analysis of the bioluminescence images (Figure 7-9) showed that in porous media A and B, biofilm tended to grow preferentially against the pressure gradient from the inoculation zone toward the inlet rather than downstream regions, whereas in porous medium C biofilm predominantly spread to downstream regions. Another interesting feature of bioluminescence images of porous media A and B was emergence of dark zones in regions previously shown to be occupied by biofilm. Therefore, to confirm biofilm spread in porous media and determine applicability of bioluminescence images in representing actual dispersion of biofilm, assays were carried out on porous media collected from upstream and downstream regions to determine bacterial cell population and protein concentration within the chambers. The results of these assays are summarized in Figure 7-10 and 7-11.

Using the porous media samples collected from porous medium C, compared to downstream regions, negligible cell concentrations were measured in upstream zone. This observation was in consistent with bioluminescence activity images of HK44 recorded
Figure 7-10: Number of bacterial cells in porous media collected from upstream and downstream sampling regions at the end of flow experiments. Values are means ± standard deviations (error bars) of 12 samples obtained from each region.

Figure 7-11: Protein concentrations measured in porous media collected from upstream and downstream sampling regions at the end of flow experiments. Values are means ± standard deviations (error bars) of 12 samples obtained from each region.
for porous medium C, in which highest bacterial bioluminescence was detected at the vicinity of the inoculation zone and more bioluminesce activities were observed at downstream regions. Such a biofilm growth pattern could be explained by moderately high bacterial detachment rates in such porous structure. The detached bacterial cells from the upstream zones can then be carried by the fluid flow and may reattach at downstreams, thereby leading to biofilm spread in the flow direction. In porous media A and B, although HK44 population was more abundant in upstream zones (which was also consistent with bioluminescence images), quantification of bacterial cells indicated relatively high cell densities within downstream regions even though these regions appeared dark in images captured following Day 5 (Figure 7-9). To verify that cells within these particular dark regions had retained their bioluminescence capability, cells in the porous medium sample collected from these regions at the end of the flow experiment were cultivated in fresh growth medium culture. Bioluminescence activities observed after exposure to induction medium confirmed the presence of active lux-genes in cells obtained from dark regions (Figure 7-9). This indicated that the lack of bioluminescence in dark regions were not due to inactivation of lux-genes, but rather the absence of factors that induce bioluminescence. Protein measurements, however, revealed a different pattern in the different porous media and were not correlated directly to the bacterial cells populations (Figure 7-11). Protein assessment in porous media samples revealed that, regardless of the biofilm sample, the average intracellular protein content of a bacterium was $1.10 \times 10^{-13}$ g of protein per cell. Therefore, the proportion of extracellular protein (i.e. EPS protein) could be estimated using the determined number of cells in each sample (Figure 7-10). In both porous media A and B, ratios of protein to bacterial population
were higher in downstream regions compared to upstream. Conversely, in porous medium C, ratio of protein concentration to cells number was similar at upstream and downstream regions.

Since total protein was measured in porous media and given that protein is present in large quantities in bacterial EPS (Nielsen et al. 1997; Dignac et al. 1998; Zhang and Bishop 2003; Metzger et al. 2009; Flemming and Wingender 2010), different ratios between protein concentrations and numbers of cells could be explained by dissimilar biofilm structures (with respect to cell density and EPS content) in samples collected from different regions of porous media. It is reported that, in poor environmental conditions, bacteria tend to spend their energy secreting more EPS rather than proliferate to protect themselves against environmental stresses (Flemming and Wingender 2010). Thus, the high ratio of EPS to cell count in downstream regions of porous media A and B could be explained by adverse growth conditions in terms of nutrient availability. Bioclogging of upstream regions extensively impede penetration of nutrients and growth factors to downstream regions, thereby establishing stressful conditions known to stimulate EPS production. In the same way, linked protein concentrations and cell numbers in porous medium C implied identical growth conditions throughout the porous medium.
7.5.4 *Tracer Experiments*

Bioclogging as a consequence of biofilm formation, can significantly impact transport properties of a porous medium, thereby influencing fluid flow and mass transfer in porous structures. On the other hand, biofilm development, as an example of a reactive process, is controlled by availability of nutrients carried by the fluid within the porous media. This indicates that, biofilm formation in porous media, which can result in bioclogging, can influence its own activity and spatiotemporal development in porous structures. Here, in order to study bioclogging in flow experiments, flow fields in porous media were further analyzed by conducting tracer experiments. After capturing each bioluminescence image, tracer solution was injected through the injection port via a three way valve to visualize the flow path of glucose solution (i.e. main carbon source) within the porous media. As illustrated in Figures 7-12, in initial tracer experiments performed on day 1, tracer plumes were transported in a straight line and uniform flow fields were observed in the entire porous media, indicating that the flow chambers were homogenously packed with glass beads with no preferential flow paths. However, in porous medium A with the finest average bead size, initial tracer experiments revealed that the tracer plume was not entirely symmetric. Thus, it could be inferred that imperfect packing could produce small-scale discrepancies in permeability of such low permeable porous medium.
Figure 7-12: Tracer movement in porous media A, B, and C during the course of 12 days experiment, indicating flow path of glucose and induction medium within porous media.
As the biofilm developed in porous media A and B, the streamlined flow of nutrient-carrying water as visualized by the tracer plumes, split when it came close to bioclogged zones, thereby resulting in the formation of two fingers around the biofilm region (Figure 7-13). Therefore, rather than convection, mass transfer was dominated by diffusion and dispersion within the biofilm-saturated regions and thus, penetration of solutes into the bioclogged sections was reduced and redirected around these low permeability regions,

Figure 7-13: Effect of biofilm on fluid flow in porous media with different grain sizes. In porous media with fine grain sizes (A and B) biofilm development can result in complete bioclogging, preventing penetration of fluid through biofilm regions. However, in porous medium with coarse beads (C), due to the moderately large pore sizes and high detachment rates, biofilm accumulation cannot continue to such extent that prevent flowing fluid from penetrating into biofilm regions.
thereby not reaching the cells in sufficient quantities. This can also have major impacts on bacterial bioluminescence within the bioclogged zones. During HK44 induction, it was likely that induction medium bypassed these bioclogged regions and instead, penetrated more permeable zones. Consequently, lack of induction agent within bioclogged zones could lower HK44 bioluminescence and thus, negatively impact the ability to visualize cells in these regions by using the over mentioned induction method. Same mechanisms can be responsible for the observed biofilm growth pattern in porous media A and B. In porous media with fine pore sizes, biofilm growth can significantly modify porous structure, resulting in major changes in hydraulic properties (Figure 7-14A). Due to bioclogging and subsequent drop in nutrient delivery to biofilm saturated regions, biomass production occurs first and foremost at the periphery of the biofilm and not at the regions surrounded by the biofilm itself (Figure 7-14A). This can lead to biofilm expansion toward the nutrient source to benefit from more favorable growth conditions with fresh and higher concentrations of nutrients. As well, reduced fluid flow through the bioclogged regions not only lowers detachment rate of microbial cells, but also enhances cell entrapment and restricts dispersion of cells in downstream regions.

No finger formation was observed during tracer experiments in porous medium C. However, the influence of biofilm on the flow field could be detected close to the chamber outlet after day 5. Biofilm formation in downstream regions slightly increased transverse dispersion in porous media and the tracer plume tended to encircle the whole biofilm regions (Figure 7-13), but the biofilm accumulation in pore spaces did not continue to such extent that prevent tracer plume from penetrating into biofilm regions.
Figure 7-14: Biofilm development can have different effects on the permeability of a porous medium depending on the size of the pores. Shown here are illustrations of porous media with small (A) and large (B) pores. In porous media with fine particles, small pore sizes result in complete biofouling, thereby impeding biofilm development at downstream regions. However, in porous media with moderately coarse particles, due to higher detachment rates, biofilm cannot completely plug pore spaces, resulting in biofilm dispersion at downstream regions.

Additionally, as illustrated in Figure 7-14B, formation of thick biofilm in large pore spaces results in higher bacterial detachment. Therefore, a steady state biofilm thickness may be reached in such porous structures. Additionally, detached cells may be carried by the flowing fluid and reattach to porous matrix, thereby resulting in biofilm spread at downstream regions.
The obtained results can help to better understand biofilm behavior in porous structures, thereby facilitating prediction, design, and operation of processes that rely on biofilms to achieve a process goal, such as wastewater treatment, in situ bioremediation, and enhanced oil recovery. According to the experimental results, pore size distribution was found as an important parameters influencing biofilm development in porous media. In addition, it was interpreted that biofilm growth pattern can be estimated based on the pore size distribution of porous media. However, effective prediction of biofilm spread in porous structures should also consider other influencing factors, such as fluid flowrate, microbial growth rate, rate of EPS production, and microbial detachment rate. In natural systems such as soil, oil reservoirs, wetlands, and other subsurface environments, porosity and pore sizes are usually smaller than what can be found in porous media composed of well sorted particles of uniform shape. According to Jorden and Campbell (1984), pore size distribution of a porous medium is not only a function of particle sizes, but also of their shape and degree of sorting. In porous media, the average pore size decreases as sorting becomes poorer (small grains fill in voids between large grains) and increases as sphericity and roundness of particles decreases (Jorden and Campbell, 1984). Therefore, as natural systems can be considered as porous media with fine pore sizes, results of porous medium A in this study can possibly be used to engineer biofilm spatial development in field-scale industrial and environmental applications.
7.6 Conclusions

By using bacterial bioluminescence, biofilm development in porous media was monitored in two-dimensional flow field. A flow chamber was designed and constructed to facilitate measuring hydraulic pressures across the porous media and by recording the fluid fluxes, dynamic variations in porous media permeability studied. Flow chambers were filled with three translucent porous media with different geohydrological characteristics. Most significant reduction in permeability (up to 59%) was observed in porous medium with smallest bead sizes, whereas in porous medium with coarse beads, permeability profile remained practically unchanged during the course of 12 days experiment. In addition, different growth patterns were observed in porous media. In porous media with fine beads, biofilm spatial development against pressure gradient (i.e. toward nutrient injection port) was linked to bioclogging of miniature pores. Likewise, the possibility of formation of thick biofilms in moderately large pores, which promotes bacterial detachment, was assumed to be the main influencing factor resulting in biofilm spread at downstreams of initially inoculated regions in porous media with coarse beads.
Chapter 8
Conclusions and Recommendations

This dissertation focused on studying the mutual interactions between biofilm growth and fluid flow in porous media. The major contributions of this thesis are summarized here followed by recommendations for future studies.

8.1 Conclusions
Biofilm formation can significantly impact performance of processes relying on fluid flow in porous structures by altering key geohydrological properties, such as permeability and mass transfer, which in turn can influence its own spread and activity within the pore networks. Therefore, controlling and engineering biofilms through an active management of their evolution in porous media can significantly improve performance of processes that are affected by biofilms. Here, in this dissertation, biofilm development in porous media was extensively studied both theoretically and experimentally to obtain more comprehensive knowledge regarding such mutual interactions between biofilm growth and fluid flow in porous media. Results from this study can be used to better understand how biofilm evolve in porous media and how biofilm development can impact flow processes in porous structures. Also, this work could serve to better predict, and possibly even control and engineer biofilm behavior in porous media.
The following conclusions were made:

1. Biofilm can be treated as a high viscosity liquid that interacts hydraulically with an aqueous phase within a porous medium. This conclusion was supported through a novel mathematical model which generated results that compared well against two-dimensional experimental observations. This is an important conclusion as a survey of the current literature will reveal that biofilm is typically treated as an evolving solid. It was revealed that biofilm (highly viscous phase) and the flowing aqueous phase can be hydraulically coupled using relative permeability functions. The relative permeability concept permitted the modeling of bioclogging effects within the biofilm rich regions. Under this approach, the flow of each phase was restricted by the presence of the other phases. More explicitly, application of relative permeability functions implied that biofilm accumulation in the pore space progressively occupies available void space and thus, restricts flow of the aqueous phase (i.e. bioclogging) and can eventually drop the water relative permeability to zero.

2. Modelling attempts and sensitivity analyses showed that interactions between water flow and movement of biofilm within the porous structure, as applied by relative permeability functions, is complicated and has significant impact on biofilm spatiotemporal development in porous media.

3. Experimental results revealed that a sensitive CCD camera in conjunction with an inducible bioluminescent bacterium can be used to monitor, in real-time, biofilm development in a translucent porous medium.
4. From real-time visualization of biofilm spatiotemporal development in packed bed of fine glass beads, it appeared that in a one-dimensional flow field, bacteria tend to grow and form biofilm preferentially from the inoculation site towards the inlet (i.e. against the pressure gradient) rather than downstream regions.

5. Under one-dimensional flow conditions, high ratios of EPS to number of bacterial cells were observed downstream of the initially inoculated sites which correlated to a lack of nutrients in such regions due to bioclogging of the upstream zones.

6. Results indicated that the detected bioluminescence intensities, calculated through image analysis, were linked to bacterial cell density, porous medium permeability, and biofilm saturation (i.e. fraction of pore space occupied by biofilm).

7. Based on the obtained results, power functions with similar allometric scaling exponents were found to correlate bioluminescence intensity to bacterial cell density in planktonic culture and biofilm. Also, results indicated that detected bioluminescence intensities were correlated as a power law to biofilm saturation and the relationship between permeability and bioluminescence intensity appeared to follow an exponential trend.

8. Real-time evaluation of biofilm saturation and porous media permeability facilitated development of relative permeability expression based on biofilm saturation in porous medium.

9. Bacterial breakthrough studies in porous media showed that formation of biofilm on solid particles can alter hydrophobicity of solid surfaces, thereby leading to
significant enhancement in bacterial sticking efficiency (enhancement as high as 91% was achieved when porous medium was covered by biofilm).

10. Good agreement between model predictions and the observed bacterial breakthrough curves indicated that the colloidal filtration theory can be used to assess bacterial sticking efficiencies in porous media.

11. Under two-dimensional flow conditions, diverse biofilm growth patterns were observed in porous media with different bead sizes. Pore sizes were found to be an important parameters influencing biofilm evolution in porous media. It was concluded that biofilm growth within the tiny pore spaces of porous media with fine beads can result in complete bioclogging, thereby restricting further biofilm development by preventing penetration of nutrients into biofilm saturated regions and corresponding downstream sections. However, availability of fresh nutrients at upstream zones leads to biofilm expansion toward the source of nutrients. On the other hand, as confirmed through tracer experiments, complete bioclogging could not be achieved at any point in porous media with coarse particles. Furthermore, formation of moderately thick biofilm in porous media with large pore sizes motivates detachment of biofilm bacteria, which can reattach to solid matrix at downstream zones. Availability of nutrients to the attached bacterial cells at downstream regions can result in biofilm dispersion at such regions.
8.2 Recommendations for Future Research

8.2.1 Relative Permeability Curves

Modelling attempts revealed that coupled flow of aqueous phase and biofilm spread, as described by relative permeability functions, is complex and can significantly affect biofilm development in porous media. The major impacts that relative permeability functions can have on biofilm model predictions imply that more intensive studies are required to better understand interactions between fluid phases in porous structures. Theoretical studies should be conducted to investigate the influence of porous media characteristics (such as porosity and pore size distribution) on degree of bioclogging, maximum biofilm saturation (i.e. fraction of pore space occupied by biofilm), and dynamic behavior of relative permeability versus biofilm saturation curves. This also requires planned experimental studies, to support theoretical rationalizations.

8.2.2 Biofilm Density

It has been shown that occupation of pore space by biofilm, which is referred to as biofilm saturation, can modify critical porous characteristics including porosity and permeability. Therefore, experimental approaches should be developed to quantify in situ biofilm volumes. Future research should focus on finding a way to quantify biofilm (bacteria and especially EPS) volume directly within porous media or indirectly determine it by measuring porosity reductions. Available and well established techniques, such as NMR and soil-water retention curves may be modified to accomplish such investigations.
8.2.3 Biofilm Spatial Development

In this study, experimental results revealed that pore size distribution is one of the main factors affecting biofilm spatial development within porous media. However, effects of other influencing factors, such as bacterial growth/decay rates, bacterial attachment/detachment rates, rate of EPS production, biofilm sloughing, and fluid velocity within the pore networks should also be investigated to obtain deeper insight into processes affecting biofilm distribution in porous structures.

8.2.4 More Realistic Conditions

In the present research, an engineered bioluminescent bacterium was used to visualize biofilm evolution in porous media. Therefore, translucent glass beads were used to minimize negative impacts of porous media on emitted bioluminescence. Correlations were developed based on the detected bioluminescence intensities to nondestructively assess biofilm and porous media properties in real-time. Such correlations, however, should be reevaluated under more realistic conditions with natural culture or consortia of microorganisms. Also, experiments should be performed using a replica of real porous media (such as consolidated porous structures) to investigate the difference between artificial and natural porous media. In addition, development of field scale applications is the ultimate goal of this biofilm research. Therefore, to validate the obtained laboratory and modelling results, biofilm growth and bioclogging should be studied in natural systems and engineered processes. As the biofilm investigation in the field requires porous media sampling with high three-dimensional spatial resolution, attempts should be made to develop appropriate and robust field sampling methods.
8.2.5 Further Investigations by 2D Flow Chamber

Although the constructed flow chamber exhibited certain complexities, it resembled a two-dimensional flow condition in which biofilm spread and flow field could be easily observed in real-time. Therefore, proposed experimental setup can be used for further porous media based biofilm investigations. Experiments can be conducted to better understand interaction between porous media heterogeneity, biofilm spatiotemporal development, and bioclogging. Also, factors influencing bioclogging, either supporting or preventing, such as chemical concentrations, fluid flowrates, biocides, porosity, pore size distribution, and physical properties of solid particles can be investigated.


232


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Appendix A

Preparation and Maintenance of Stuck Cultures

*Pseudomonas fluorescens* HK44 was received as freeze-dried samples and used to prepare stock cultures. Development of a viable stock culture was necessary to ensure a continued supply of original culture throughout these studies. Preparation of a stock culture includes growing of microorganisms in an appropriate growth medium, aliquoting the microbial suspension into small cryovials, freezing the samples, and keeping the vials in a freezer at -80°C. All the subsequent cultures were inoculated directly from this frozen stock to ensure genetic similarity.

A-1- Preparation of Stock Culture Growth Medium

The growth medium used in all biofilm experiments was made of four solutions: glucose solution (as the main carbon source), mineral based solution, trace element solution, and tetracycline solution. Composition of each solution is detailed in Table A.1. The pH of each stock solution was adjusted to 7.00 ± 0.05 with 1 M NaOH or HCl. The solutions were prepared using distilled water. As glucose decomposes during autoclaving, glucose, mineral based, and phosphate buffered saline solutions were autoclaved separately and sterilized stock solutions were stored at 4°C for up to two weeks. To prepare 1.0 liter of growth medium, 333 mL of sterilized glucose, mineral based, and phosphate buffer saline solutions were combined and 1 mL of tracer and tetracycline solutions were added to the
Sterile growth medium using a disposable sterile 0.2 μm syringe filter. In addition, an induction medium was prepared to induce the lux genes, by adding 0.1 g/L (final concentration) of salicylate and removing all phosphate sources from the growth medium.

**Table A-1: Composition of solutions used to prepare growth medium.**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Solution</td>
<td>Glucose</td>
<td>3 (g/L)</td>
</tr>
<tr>
<td>Mineral Based Solution</td>
<td>MgSO₄</td>
<td>1.2 (g/L)</td>
</tr>
<tr>
<td></td>
<td>CaCl₂.2H₂O</td>
<td>0.3 (g/L)</td>
</tr>
<tr>
<td></td>
<td>NH₄Cl</td>
<td>1.2 (g/L)</td>
</tr>
<tr>
<td>Phosphate Buffered Saline Solution</td>
<td>NaCl</td>
<td>24 (g/L)</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>0.6 (g/L)</td>
</tr>
<tr>
<td></td>
<td>NaH₂PO₄</td>
<td>3.45 (g/L)</td>
</tr>
<tr>
<td></td>
<td>K₂HPO₄</td>
<td>0.78 (g/L)</td>
</tr>
<tr>
<td>Trace Elements Solution</td>
<td>HCl</td>
<td>3.66 (g/L)</td>
</tr>
<tr>
<td></td>
<td>FeSO₄.7H₂O</td>
<td>21 (g/L)</td>
</tr>
<tr>
<td></td>
<td>H₃BO₃</td>
<td>0.3 (g/L)</td>
</tr>
<tr>
<td></td>
<td>MnCl₂.4H₂O</td>
<td>1 (g/L)</td>
</tr>
<tr>
<td></td>
<td>CoCl₂.6H₂O</td>
<td>1.9 (g/L)</td>
</tr>
<tr>
<td></td>
<td>NiCl₂.6H₂O</td>
<td>0.24 (g/L)</td>
</tr>
<tr>
<td></td>
<td>CuCl₂.2H₂O</td>
<td>0.02 (g/L)</td>
</tr>
<tr>
<td></td>
<td>Na₂EDTA.2H₂O</td>
<td>10 (g/L)</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄.7H₂O</td>
<td>1.44 (g/L)</td>
</tr>
<tr>
<td></td>
<td>Na₂MoO₄.2H₂O</td>
<td>0.36 (g/L)</td>
</tr>
<tr>
<td>Tetracycline Solution</td>
<td>Tetracycline</td>
<td>30 (g/L)</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>50 % (v/v water)</td>
</tr>
</tbody>
</table>
A-2- Preparation a Stock Culture from Freeze-Dried Cells

The HK44 cells were retrieved from the freeze-dried sample at 27°C in Luria broth (LB) medium containing 5 g NaCl, 5 g yeast extract, 10 g tryptone, and 15 mg tetracycline per 1 L distilled water (pH 7.0). The procedure used to prepare a stock culture of HK44 had two steps:

**Step one – retrieving HK44:**

1. Break the tip of the ampoule containing the cells (heat the tip and drip two-three drops of water to crack the glass)
2. Add 0.3 - 0.4 mL of LB medium using a sterile syringe.
3. Transfer the mixture into a sterile 10 mL test tube filled with 5 mL LB medium.
4. Incubate the cells overnight at 27°C.

After 24 hours, when visible growth can be detected, use the retrieved cells to prepare stock culture.

**Step two – Stock culture preparation:**

1. Fill a 250 mL sterile conical flask with 100 mL of autoclaved growth medium.
2. Inoculate the medium with 10 mL of retrieved HK44 and close the flask with sterile cotton and wrap it with aluminum foil.
3. Place the flask on a rotary shaker operated at 150 revolutions (rpm) and let bacteria grow overnight at room temperature.
4. Prepare cryogenic vials by labeling them (type of microorganism, date, and name of researcher).
5. Prepare a solution of 20% glycerol (v/v) by mixing 20 mL of glycerol with 80 mL of water.

6. Transfer the solution to a screw cap bottle and sterilize by autoclave at 121°C for 15 minutes (loosen the cap during autoclaving).

7. Harvest the cells by centrifuging the grown bacterial culture.

8. Pour off the supernatant and add 50 mL of the 20% glycerol solution to the harvested cells.

9. Mix them by using vortex mixer.

10. Aliquot 1 mL of final culture solution into cryogenic vials.

11. Store the vials in the -80°C freezer.

To recover frozen cells for cultivation, the vials were quickly thawed in a 37°C water bath. When needed, individual vials were removed from the freezer, thawed, and inoculated into freshly prepared LB medium. Following overnight cultivation at 30°C, 0.5 mL of the growing culture was inoculated into 40 mL of fresh growth medium and after 24 hours, cells were harvested by centrifugation at 10,000 rpm for 20 minutes, washed in phosphate buffer saline solution, centrifuged again and dispersed in induction medium to a density of 5×10^8 cells/mL to confirm bioluminescence activity. This bacterial suspension was used in biofilm experiments.
Appendix B

Evaluation of Biokinetic Parameters

Biokinetic parameters characterizing the growth of planktonic bacteria in suspension should be evaluated prior to conducting any biofilm experiment. Such information facilitates modelling microbial growth and simulation of biofilm formation in porous media. A Monod kinetics expression predicts the microbial growth rate as a function of a single growth-limiting nutrient as following:

\[
\frac{dN}{dt} = \mu_{\text{max}} \frac{S}{K_S + S} N
\]  

(B.1)

where \( N \) is the cell number, \( t \) is time, \( \mu_{\text{max}} \) is the maximum specific growth rate, \( S \) is growth-limiting substrate, and \( K_S \) is the half saturation constant.

However, when the nutrient concentration is in excess (\( S >> K_S \)), then equation (B.1) simplifies to:

\[
\ln N = \mu_{\text{max}} t + \ln N_0
\]  

(B.2)

Therefore, the maximum growth rate can be determined from a linear regression analysis of the slope of the logarithmic phase of growth. Considering the HK44 growth curve and by using Equation (B.2), the HK44 maximum specific growth rate constant was calculated as 0.215 h\(^{-1}\).
Figure B-1: Growth curve of HK44 in growth medium used to calculate maximum growth rate coefficient.
Appendix C

Porous Media Sampling

Biofilm in porous media can be difficult to analyze. To determine the number of cells or protein concentration, the biofilm should be removed from the porous media and disaggregated into a homogeneous cell suspension.

Sonication was found to be the best method to detach biofilm from porous media and also to disaggregate the individual cells from the EPS. Preliminary efforts revealed that, vortexing alone, was not able to sufficiently disaggregate biofilm, leaving several biofilm clusters intact. However, due to the exposure of high intensity ultrasound and the detrimental impacts that a sonication probe can have on viable cells, utilization of an ultrasonic bath would be more appropriate to maintain cells viability and activity.

The following steps were followed to detach biofilm from porous media samples and disaggregate the bacterial cells from the biofilm clusters:

1. The porous media samples were placed into test tubes containing desired volume of diluents (e.g. PBS) to resuspend and disaggregate biofilm.
2. Tubes were vortexed for one minute on the highest power.
3. Ultrasonic bath was filled with water and the tubes containing diluent and porous media were placed in it.
4. Vortexed samples were sonicated for 30 seconds.
5. Each sample was vortexed again for one minute.
6. Each sample was sonicated again for 30 seconds.

Obtained biofilm samples were ready for further analysis such as cell enumeration and protein measurement.
Appendix D

Preparation of Biofilm Samples for Biological Assays

Following protocol was used to prepare biofilm samples for further biological analysis:

1. Sonicate the biofilm samples for 30 seconds at the highest power using a sonicator probe.
2. Aliquot 1.5 mL of biofilm suspension into separate microcentrifuge tubes.
3. Freeze the aliquots at -80°C for one hour.
4. Put the frozen samples into a beaker filled with ice to keep the cool.
5. Sonicate each aliquot twice at high power using a sonicator probe for 30 seconds and rest for 30 seconds in between to allow the samples and the probe to cool down. Before increasing the power and all over the sonication, probe must be immersed in the aliquot. In addition, probe should not touch the bottom or sides of the microcentrifuge tube as it can be melted, result in losing the sample. In addition, rising up the sonicator power unconsiously can cause the sample to boil out of the microcentrifuge tubes.
6. Centrifuge each microcentrifuge tube for 5 minutes at high speed to pellet bacterial cells debris.
7. Supernatant can be pipetted into a clean labeled vial and be used in biological assays.
Appendix E

Absorbance Standard Curve for HK44 Evaluation

Figure E-1: Standard curve used to correlate HK44 population density to the light absorbance at 550 nm. Values are average absorbance with error bars based on five replicates for each cell concentration.

\[ y = 6 \times 10^{-10}x + 0.0012 \]
\[ R^2 = 0.997 \]
Appendix F

DNA Assay

The DNA assay kit used in this study was based on the selective binding of DNA to silica-based membrane in the presence of chaotropic salts. The lysate was prepared from biofilm samples. Bacterial cells were digested with Proteinase K at 55°C by using an optimized digestion buffer to denature proteins and enhance Proteinase K activity. Also, to minimize contamination of RNA, any residual RNA was removed by digestion with RNase A prior to binding samples to the silica membrane. After binding DNA to the silica-based membrane, impurities were removed by washing with a buffer solution and finally, the genomic DNA was eluted in low salt elution buffer (10 mM Tris-HCL- pH 9.0 and 0.1 mM EDTA) to increase DNA yield. The following protocol gives instruction on lysing bacterial cells and purification of DNA in the obtained lysate:

Preparation of lysate:

1. Set the water bath at 55°C.
2. Harvest bacterial cells by centrifugation (higher bacterial concentration results in more accurate readings).
3. Resuspend harvested cell pellet in 180 μL Digestion buffer.
4. Add 20 μL RNase A to the lysate, mix well by brief vortexing, and inoculate at room temperature for 2 minutes (vortexing in each step should be less than 5-10 seconds to prevent extensive shearing of DNA).
5. Add 200 μL Lysis/Binding buffer and mix well by vortexing to obtain a homogeneous solution.
6. Add 200 μL high concentration ethanol (> 96%) to the lysate. Mix well by vortexing for 5 seconds to yield a homogeneous solution.

**Binding DNA:**

1. Centrifuge the lysate at 10,000 rcf for 1 minute at room temperature using the provided spin columns.
2. Discard the supernatant and place the spin column into a clean collection tube containing silica-based membrane.
3. Add 500 μL Washing buffer prepared with ethanol to the collection column.
4. Centrifuge the solution at 10,000 rcf for 1 minute at room temperature.
5. Repeat steps 3 and 4.

**Eluting DNA:**

1. Place the collection tube in a sterile 1.5 mL microcentrifuge tube.
2. Add suitable volume of Elution buffer to the tube.
3. Incubate at room temperature for 1 minute.
4. Centrifuge the tube at maximum speed for 1 minute.
5. Repeat steps 2 to 4 to improve DNA recovery.

The tube containing purified DNA can be stored at -20°C for long term storage or used immediately to determine DNA concentration.
To determine DNA concentration following procedures should be followed:

1. Use two thin-wall, clear 0.5 mL PCR tubes for the standards and one tube for each DNA sample.
2. Label the tube lids.
3. Add provided dye solution (Qubit® assay) to each tube (the advanced dyes only fluoresce when bound to DNA) and dilute the DNA suspension using the abovementioned dilution buffer.
4. Vortex all tubes for 2-3 seconds.
5. Incubate the tubes for 2 minutes at room temperature.
6. Calibrate the fluorometer using the prepared standard samples.
7. Put the tubes in the fluorometer and take reading.
Appendix G

Mass of DNA versus HK44 Cell Numbers

Figure F-2: Regression analysis revealed strong linear correlation between mass of DNA and number of HK44 cells. The obtained correlation can be used to estimate HK44 population based on DNA measurements.
Appendix H
Protein Assay

Biofilm samples (bacterial cells and the associated EPS) contain considerable amount of protein. Thus, quantification of total protein provides more practical estimation of the amount of biofilm rather than precise enumeration of individual biofilm associated cells.

To lyse remaining bacterial cells in biofilm samples (see Appendix C) bacterial protein extraction lysis buffer (Bacterial-PELB) was used to extract all intracellular proteins. Bacterial-PELB is based on organic buffering agents and utilizes a mild non-ionic detergent to enhance extraction and stability of proteins. This extraction method is an improvement on the lysozyme based lysis, which extracts all soluble proteins and concurrently removes all nucleic acids (DNA and RNA) released during cell lysis. Therefore, eliminates the need for mechanical lysis of bacterial cells and removal of DNA/RNA with nuclease treatments.

Following procedures were followed to extract intracellular protein and concurrently remove nucleic acids:

1. Pellet bacterial cells by centrifugation of 250 µL biofilm samples at 10,000 rcf for 10 minutes.
2. Resuspend the cell pellet in 250 µL Bacterial-PELB buffer.
3. Vortex the for 1 minute to homogenize the bacterial suspension.
4. Incubate the suspension for 5 minutes in cold water bath and vortex again to suspend the cells.
5. Add 5 μL Lysoszyme for each 100 μL cell suspension and vortex for 1 minute.

6. Incubate the mixture for 45 minutes at 37°C.

7. Vortex the mixture 3 to 5 times to complete lysis. Lysis can be further assisted by pipetting the suspension via a narrow bore pipette or a syringe needle.

8. Centrifuge the lysate at 20,000 rcf, 4°C for 30 minutes and collect the clear lysate.

Lysate is now ready for protein assay. The BCA (Bicinchoninic Acid) protein assay was then used to determine protein concentration in lysate. The BCA protein assay is based on colorimetric detection and quantification total protein using Bicinchoninic Acid. This method combines biuret reaction (reduction of Cu$^{2+}$ to Cu$^{1+}$) by protein in an alkaline medium with the selective colorimetric detection of monovalent copper ions (Cu$^{1+}$) by using a unique reagent containing bicinehnonic acid. The purple reaction product of this assay is formed by binding of two bicinchoninic acid molecules with one monovalent copper ion. The obtained water-soluble reaction complex exhibits a strong absorbance at 562 nm which linearly increases with protein concentration in the board range of 20-2000 μg/mL.

Protein concentrations can be evaluated with reference to standards of a common protein, such as bovine serum albumin (BSA). Therefore, to setting up a protein standard curve, a series of dilutions of known concentration of BSA were prepared from the protein and assayed before conducting assay on biofilm samples (Figure H-1).

The following procedures describe the assay protocol used to determine biofilm samples protein content:
1. Pipette 100 μL of biofilm sample into labeled tube.

2. Add 1 mL BCA solution, composed of bicinchoninic acid, sodium carbonate, sodium tartate, cupric sulfate, and sodium bicarbonate in 0.1 M NaOH, and vortex for 10 seconds.

3. Incubate the tube in a water bath at 60°C for 15 minutes.

4. Allow tubes to cool to room temperature. As color development continues after cooling to room temperature, the cooling interval should be kept consistent in all measurements.

5. Add 1 mL BCA solution to a clean spectrophotometer cuvette and adjust the absorbance reading at 562 nm to zero.

6. Add 1 mL of incubated solution to a clean cuvette and measure and record the absorbance at the same wavelength.

7. Use the protein standard curve (Figure H-1) to determine protein concentration of the biofilm sample (the absorbance (i.e. protein concentration) should fall within the linear range of the standard curve).

8. Calculate the actual protein concentration of biofilm sample by correcting for the dilution and sample volume.
Figure H-1: Standard curve used to determine protein concentration of biofilm samples.

\[
y = 0.0018x + 0.0553
\]

\[
R^2 = 0.9891
\]
Appendix I

Supplementary Bioluminescence Images

Figure I-1: Bioluminescence images obtained on Day 8 for two replicates of the one-way induction experiment that was described in the main text (Figure 4-3). It is evident that similar bioluminescence activities were observed in all replicates of the experiment (see Figure 4-3 of the main text). In each case, bioluminescence was detected primarily in the upstream zones (Zones 1 and 2) towards the induction medium injection port. Significantly lower bioluminescence intensities were detected downstream of the initially inoculated region (in Zones 3 and 4). Scale indicates increasing bioluminescence intensity from 0 to 6.
Figure I-2: Shown here are the bioluminescence images obtained from one of the other one way induction trials. (A) Bioluminescence response of the cells indicating biofilm dispersion in porous medium over the course of 8 days. The induction medium was injected via the inlet located to the left of Zone 1, and discharged from the outlet located to the right of Zone 4. The chamber was inoculated at the interface between Zones 2 and 3. Scale indicates increasing bioluminescence intensity from 0 to 6. (B) Average number of bacterial cells in seven samples collected from each zone at the end of the experiment.
Figure I-3: (A) To show reproducibility of data in two-way induction experiments, the overall bioluminescence image obtained from a second trial is presented. The overall bioluminescence image was obtained by merging the images captured following both inlet and outlet induction on Day 8 of the experiment. Scale indicates increasing bioluminescence intensity from 0 to 6. (B) Number of cells versus average bioluminescence intensity calculated on Day 8. In Figure 4-10 from the main text, a power function relationship was derived using that set of data. The same power function relationship has been superimposed on this figure (dashed line). It is evident that the scaling exponent (equal to 3.11) obtained using the data in Figure 4-10 is also a good fit for the second set of data displayed here. (C) Flow rates through the porous medium versus overall bioluminescence intensities calculated for the entire porous medium. In Figure 4-12 from the main text, a linear relationship was derived using that set of data. The same linear correlation has been superimposed on this figure (dashed line). It is evident that the linear correlation obtained using the data in Figure 4-12 is also a good fit for the set of data displayed here.
Appendix J

MATLAB® Code for Image Processing

Reading bioluminescence image to process

IMAGE=imread('bioluminescence image.tif');
imtool (IMAGE);  % display original image

Convert bioluminescence image to gray scale

GSImage=rgb2gray(IMAGE)  %Convert to gray scale
imtool (GSImage)  % display gray level bioluminescence image
imwrite (GSImage, 'GrayScaleImage.tif'); % write gray scale image

Merging bioluminescence images

% INTRODUCE FUNCTION TO MERGE BIOLUMINESCENCE IMAGES

function a = mergpix( GSImage1, GSImage2)
A=GSImage1;  % passed first bioluminescence image
B=GSImages2; % passed second bioluminescence image
SizeA = size (A);  % dimensions of first bioluminescence image
SizeB = size (B);  % dimensions of second bioluminescence image
% NUMBER OF ROWS AND COLUMNS

nrA = size(A, 1); % number of rows in A
ncA = size(A, 2); % number of columns in A
nrB = size(B, 1); % number of rows in B
ncB = size(B, 2); % number of columns in B

% MERGE IMAGES BY SELECTING HIGHEST INTENSITIES

for i=1:nrA
    for j=1:ncA
        if (A(i,j)>B(i,j))
            B(i,j)=A(i,j);
        end
    end
end

imtool(C);

% The final image will be opened in the Image Tool window. Click Adjust Contrast button in the toolbar, or select the Adjust Contrast option from the Tools menu (Image Processing Toolbox should be included) to open a histogram of the image in a separate window. The histogram illustrates the range of intensity values actually used in the target image within the available gray levels (i.e. default display range of the grayscale colormap). Using the display boundaries and manipulating its size and position, image contrast can be enhanced by spreading the image intensity values over the whole colormap, thereby resulting in much more detail in the image.
% INTRODUCE FUNCTION TO SEGMENT BIOLUMINESCENCE IMAGE TO 4 ZONES (see Figure 4-3 for more details of each zone).

function b = pixsegmentation( GSImage)

A=GSImage;  % passed bioluminescence image

% INTRODUCE VARIABLES FOR THRESHOLD VALUES
Threshold1 = 0;
Threshold2 = 0;
Threshold3 = 0;
Threshold4 = 0;

% NUMBER OF ROWS AND COLUMNS
nrA = size(A, 1);  % number of rows in A
ncA = size(A, 2);  % number of columns in A
ncZone = idivide(ncA, 4, 'round');  % number of columns for each Zone

% INTRODUCE MATRIX FOR EACH ZONE
Zone1=zeros(nrA, ncZone);
Zone2=zeros(nrA, ncZone);
Zone3=zeros(nrA, ncZone);
Zone4=zeros(nrA, ncZone);
for i=1:nrA
   j1=ncZone;   % end of Zone 1
   j2=2*ncZone; % end of Zone 2
   j3=3*ncZone % end of Zone 3
   j4=4*ncZone % end of Zone 4
   for k1=1:j1
      Zone1(i,k1)=A(i,k1);
   end
   for k2=(j1+1):j2
      Zone2(i,k2)=A(i,k2);
   end
   for k3=(j2+1):j3
      Zone3(i,k3)=A(i,k3);
   end
   for k4=(j3+1):j4
      Zone4(i,k4)=A(i,k4);
   end
end   % for i

Threshold1=mean2(Zone1)
Threshold2=mean2(Zone2)
Threshold3=mean2(Zone3)
Threshold4=mean2(Zone4)
Calculate threshold value of gray scale bioluminescence image

% introduce threshold function to be easily called to calculate threshold value of a gray level image.

    function t=threshold(GSImage , GrayLevel)  % threshold function

    GL=GrayLeveL;    % Number of gray levels in the image

    X=GSImage;   % passed image

    d=size(X);   % calculate image size

    XHist = imhist(X);  % Create image histogram

    MatrixIndex=1:GL;

    plot(MatrixIndex,XHist(:,1))   % to see the histogram

    threshold=300;   % any number bigger than maximum gray level

% CALCULATE THRESHOLD VALUE

    for count=1: GL

        % CALCULTEAE FOREGRAOUND GRAY LEVEL

        FG=double(XHist(1:count,1));   % foreground histogram

        FGIndex=MatrixIndex(1:count,1); % foreground index

        FGOOut=FG.*FGIndex; % multiply index and histogram

        SumFG=sum(FGOOut); % summation of foreground and histogram product

        SumFGIndex=sum(FG); % sum of histogram FG

    end

271
% GET RID OF DEVISION BY ZERO

if SumFGIndex==0;

    FGGrayLevel=0;

else

    FGGrayLevel=SumFG/SumFGIndex; % foreground gray level

end

% CALCULATE BACKGROUND GRAY LEVEL

BG=double(XHist((count+1):GL,1)); % background histogram

BGIndex=MatrixIndex((count+1):GL,1); % background index

BGOOut=BG.*BGIndex; % multiply index and histogram

SumBG=sum(BGOOut); % summation of background and histogram product

SumBGIndex=sum(BG); % sum histogram

if SumBGIndex==0;

    BGGrayLevel=0;

else

    BGGrayLevel=SumBG/SumBGIndex; % average index

end
% CACULATING THRESHOLD VALUE

threshold=(FGGrayLevel+BGrayLevel)/2;

if threshold<=(count+1)

    if threshold>=(count-1)

        break;

    end % if

end % if

end % for

ThresholdValue=threshold/GL

Convert to binary (i.e. black and white) image

% INTRODUCE FUNCTION TO CONVERT GRAY SCALE BIOLUMINESCENCE IMAGE TO BLACK AN WHITE, BASED ON THRESHOLD VALUE

function c = gray2BW( GSImage)

    A=GSImage;  % passed bioluminescence image

    BWImage=im2bw(A, ThresholdValue);

    Imtool(BWImage);

end
Appendix K

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A) Biofouling Journal (TYLOR & FRANCIS)

Bozorg A., et al., 2012

Biofouling

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