Bacterial Enhancement of Contaminant Bioavailability: Effects of Random Motility and Chemotaxis

A Thesis
Submitted to the Faculty
of
Drexel University
by
Rajveer Singh
In partial fulfillment of the requirements of the degree of
Doctor of Philosophy

September 2011
Dedications

To my parents and brothers,

For their endless sacrifices and perseverance to make my way up to this point of life

To my brother Yashveer,

For keeping all the thorns of my way and presenting me only roses, without his selfless acts this work would not have been possible
Acknowledgement

This work would not have been possible without the support and encouragement of my family, friends, and mentors. I would like to take this opportunity to express my sincere gratitude especially to the following people.

I’d like to thank my advisor Dr. Mira Olson for her guidance, enthusiasm, and encouragement, and being easily accessible all the times throughout this research project. I’d also like to acknowledge my committee members Drs. Charles Haas, Richard Weggel, Markus Hilpert, and Patricia Gallagher for their suggestions and review of this work at various stages. I am sincerely thankful to Dr. Moses Noh, MEM department, for his expert guidance with microfluidics and otherwise. I’d also like to thank Dr. MinJun Kim, MEM department, for allowing me to use his labs for microfluidic work. I am thankful to Dr. Joseph Martin, Dr. Robert Brehm and other faculty members of CAEE department with whom I have had opportunity to work and interact during my stay at Drexel.

I was fortunate to have a great company of some friends and fellow graduate students—Dhruv, Arun, Asav, Shreyas, Garima, Priyanka, Neha, Shamia, Jade, Ed, Rafael, Dalhyung, Ukei, Tim, Sondra, Haibo, Sushil, Tao, just to name a few— who have helped me and made life more enjoyable in and out of the office during my stay at Drexel.

Words cannot express how grateful I am for having a great family which is the biggest asset and inspiration of my life. I’d like to express my profound gratitude to my parents, Mrs. Vikrama Devi and Mr. Shreepal Singh Rawat for their love, support, and encouragement through all phases of my life. Your belief in my abilities and inspiration to achieve the best has been a constant source of strength and stability throughout my life. I am thankful to my brothers, Yash and Satty, for sharing all the enjoyable and difficult moments and helping me to make important decisions of my life.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................ iii  
LIST OF TABLES.................................................................................................................. viii  
LIST OF FIGURES............................................................................................................... xi  
ABSTRACT............................................................................................................................ xiii  

CHAPTER 1: INTRODUCTION.......................................................................................................................... 1  
1.1. Motivation.......................................................................................................................................... 1  
1.2. Research objectives......................................................................................................................... 3  
1.3. Dissertation structure..................................................................................................................... 4  

CHAPTER 2: BACKGROUND......................................................................................................................... 6  
2. Introduction.......................................................................................................................................... 6  
2.1. Bacterial transport in soil.................................................................................................................. 6  
2.1.1. Bacterial migration mechanisms................................................................................................. 8  
2.1.1.1. Bacterial random motility......................................................................................................... 8  
2.1.1.2. Bacterial Chemotaxis............................................................................................................. 10  
2.1.2. Why microorganisms pose chemotaxis...................................................................................... 10  
2.1.3. Molecular mechanisms of chemotaxis....................................................................................... 11  
2.1.4. Random motility and chemotaxis assays.................................................................................... 12  
2.1.4.1. Capillary assay....................................................................................................................... 12  
2.1.4.2. Stopped-flow diffusion chamber (SFDC) assay................................................................. 15  
2.1.4.3. Agarose plug assay................................................................................................................. 16  
2.1.4.4. Swarm plate assay................................................................................................................ 17  
2.1.4.5. Drop assay............................................................................................................................ 17  
2.1.4.6. Three dimensional tracking microscopy............................................................................... 18  
2.1.4.7. Chemotactic index (C.I.)...................................................................................................... 18  
2.1.5. Chemotaxis transport parameters.............................................................................................. 18  
2.1.5.1. Chemotaxis sensitivity coefficient ($\chi_0$).............................................................................. 19  
2.1.5.2. Chemotaxis receptor constant ($K_d$)................................................................................... 20  
2.1.5.3. Chemotactic velocity ($v_c$).................................................................................................. 20  
2.1.6. Chemotactic bacterial transport models.................................................................................... 22  
2.1.7. Review of bacterial chemotaxis toward environmental pollutants............................................ 23  
2.1.7.1. Chemotaxis toward simple aromatic compounds.............................................................. 23  
2.1.7.2. Chemotaxis toward polycyclic aromatic compounds (PACs).......................................... 24  
2.1.7.3. Chemotaxis toward nitroaromatic compounds (NACs)...................................................... 24  
2.1.7.4. Chemotaxis toward chlorinated compounds..................................................................... 27  
2.1.8. Quantification of chemotaxis for bioremediation................................................................. 28  
2.1.8.1. Quantification of chemotaxis in bulk liquids................................................................. 28  
2.1.8.2. Quantification of chemotaxis in saturated porous media................................................. 29
4. Abstract .......................................................... 70
4.1. Introduction .................................................. 70
4.2. Materials and Methods .................................. 74
  4.2.1. Cell culture preparation and chemicals ........ 74
  4.2.2. Plug assay experiments ............................. 75
  4.2.3. Design and fabrication of Microfluidic device (MFDs) operation ... 75
  4.2.4. Microfluidic device (MFD) set up and operation for chemotaxis experiments .......................................................... 77
  4.2.5. Data acquisition and analysis ....................... 77
  4.2.6. Transverse dispersion of DL-aspartic acid (Dchy) .... 80
4.3. Results and discussion ................................... 81
  4.3.1. Plug assay experiments ............................. 81
  4.3.2. Transverse chemotactic migration of bacteria in MFD-IV ................... 82
  4.3.3. Bacterial accumulation in low permeability region .................. 86
CHAPTER 5: KINETICS OF TRICHLOROETHYLENE AND TOLUENE TOXICITY TO PSEUDOMONAS PUTIDA F1 .......................................................... 90
5. Abstract .......................................................... 90
5.1. Introduction .................................................. 91
5.2. Materials and Methods .................................. 94
  5.2.1. Live-dead bacterial viability assay ............... 94
  5.2.2. Cell culture ............................................ 95
  5.2.3. Cell staining protocol ............................... 96
  5.2.4. Live-dead chemotaxis plug assay .................. 96
  5.2.5. Toxicity assay ........................................ 97
  5.2.6. Cell enumeration ..................................... 97
  5.2.7. Growth curves ........................................ 98
  5.2.8. Data analysis ......................................... 99
5.3. Results ....................................................... 99
  5.3.1. Chemotaxis and viability of P. putida F1 in a TCE gradient .......... 99
  5.3.2. Live-dead toxicity assay ............................ 100
  5.3.3. Toxicity kinetics ...................................... 102
  5.3.4. Cell viability .......................................... 104
5.4. Discussion .................................................. 104
CHAPTER-6: FIELD-SCALE APPLICATION OF CHEMOTAXIS TO GROUNDWATER BIOREMEDIATION .................................................. 112
6. Background ..................................................... 112
  6.1. Enhanced remediation due to chemotaxis in heterogeneous porous media ... 114
  6.2. Application of chemotaxis for bioaugmentation ................. 114
  6.3. Chemotaxis in monitored natural attenuation (MNA) .................. 119
  6.4. Application of chemotaxis for contaminant containment – A variable length biocurtain .................................................. 119
CHAPTER 7: SUMMARY AND CONCLUDING REMARKS

7.

7.1. Summary and conclusions

7.1.1. Simulation of groundwater contamination scenario in microfluidic devices

7.1.2. Contaminant mixing enhancement

7.1.3. Bacterial chemotaxis

7.1.4. Toxicity kinetics of contaminant

7.2. Significance of the work

7.3. Future research recommendations/Beyond this research

REFERENCES

APPENDIX-A: DESIGN OF DUAL-PERMEABILITY MICROFLUIDIC DEVICE (MFD-IV)

APPENDIX-B: COMPARISON OF LIVE AND DEAD CELL INTENSITY PROFILES IN LIVE-DEAD CHEMOTACTIC AND CONTROL PLUG ASSAY EXPERIMENTS

APPENDIX-C: COATED MICROFLUIDIC DEVICE: A BIOCHEMICAL SENSOR FOR TRACKING BACTERIA AND CONTAMINANT TRANSPORT

C.1 FITC labeling of microfluidic devices

C.2 Concanavalin A and Glycogen coating

APPENDIX-D: EXPERIMENTAL PROTOCOLS

D.1 Micro Fabrication

D.1.1 Photolithography process for a SU8-2025 mold fabrication for MFDs

D.1.2 PDMS soft lithography process for MFD fabrication

D.2 Live/Dead Bacterial Viability Assay

APPENDIX-E: HYDROGEOLOGIC CHARACTERISTICS OF THE MICROFLUIDIC DEVICES
LIST OF TABLES

Table 2.1: Random motility and chemotactic sensitivity coefficients of different bacterial strains under different experimental conditions ................................................................. 21

Table 2.2: List of bacterial strains that are chemotactic toward various environmental pollutants .......................................................................................................................... 25

Table 3.1: Summary of apparent dispersivity ($\alpha_{app}$) values for three devices under different experimental conditions .......................................................................................... 52

Table 4.1: Hydrogeologic properties of microfluidic device (MFD-IV). .......................................................................................................................... 78

Table 4.2: Change in bacterial counts in low permeability zone at various cross-sections under chemotactic and non-chemotactic conditions. Average relative bacterial counts and standard deviation are reported for triplicate experiments ...................................................................................... 87

Figure 5.1: Best fit linear and exponential (Eq. 1 and 2, respectively) model parameters for Pseudomonas putida F1 survival data following trichloroethylene and toluene exposure, with corresponding $r^2$ values. $k$ is a toxicity constant and $m$ and $n$ represent empirical constants ............................................................................................................................... 103

Table E.1: Hydrogeological properties of the MFDs used in this study .......................................................................................................................... 157
LIST OF FIGURES

**Figure 2.1:** A typical bacterial motility mechanism consists of a series of straight runs and tumbling movements (A). The random motion of the bacteria results in chaotic movement where overall motion looks like a 3D-random walk similar to the diffusion of gaseous molecules (B). Under the influence of chemical gradients bacteria bias their random motion, either toward or away from the source, and the resulting motion looks like a biased random walk (C). ........................................................................................................................................................................... 9

**Figure 2.2:** Schematic representing a possible evolution in the bacterial chemotactic response to environmental pollutants as a result bacterial pursuit for carbon and energy resources. Improved understanding of this acquired behavioral response of degradative bacteria can help to design enhanced *in-situ* bioremediation strategies................................................................. 13

**Figure 2.3:** Bacterial chemotaxis assays. Swarm plate assay (panel A): A growth medium with low agar content is poured into a Petri dish. Motile bacteria are stabbed in the middle. A sharp chemotactic band moving outward from inoculation point can be observed (A(i)) in as comparison to blank (A(ii)). Drop Assay (panel B): Similar to swarm plate assay except bacteria are suspended in drop assay medium and attractant is placed at the centre of Petri dish. Chemotactic bacterial band can be observed around the attractant (B(i)) in comparison to control (B(ii)). Capillary assay (panel C): Attractant, placed in capillary tube diffuses out in pool of motile bacteria. In response to chemical gradient cells accumulate at the mouth of capillary which can be observed under microscope. Agarose plug assay (panel D): attractant is mixed with melted agarose plug in the middle of chamber formed by cover slip and plastic strips; and bacterial suspension is flooded into chamber. Chemotactic band can be visualized under microscope........................................................................................................................................................................... 14

**Figure 3.1:** Schematic representing the bi-layer micro-fluidic device (MFD) used for testing contaminant mixing enhancement due to bacterial motility in porous media. An isometric-view (top) showing the porous channel (between the glass and first PDMS layers) simulating a very thin layer of a two dimensional aquifer slice connected to an overlying channel (between two PDMS layers) simulating leakage from an underground storage tank. A vertical cross-sectional view through section AA’ (bottom) shows the closed channel opening though which different injectates flow through the device. Cross-sections representing sampling locations at 4.9, 9.8, 19.6, 29.2, and 36.75 mm downstream from the tracer inlet point into the porous channel are also shown (top). A representative zoomed-in view at a cross-section is also shown on the right. Specific details of different devices are presented in Table 3.1........................................................................................................................................................................... 48

**Figure 3.2:** Images showing zoomed-in pore geometry details of the three pore designs used in this study- a regular pattern of uniform grain size (MFD-I), a regular pattern of two different grain sizes (MFD-II), and a regular pattern of uniform grain size with equivalent porosity of MFD-II (MFD-III). Bottom right side picture shows a macroscopic image taken at 63X magnification inside a pore body of MFD-II. The larger grain diameter in all devices (dark black circles in A, B, and C) is 0.300 mm and smaller grain diameter in MFD-II (smaller black dots in B) is 0.100 mm. Pore throat spacing in MFD-I and MFD-II is 0.050 mm and in MFD-III is 0.032 mm........................................................................................................................................................................... 52

**Figure 3.3:** Typical fluorescein isothiocyanate labeled dextran (FITC-Dextran) dispersion images at cross-sections 9.6 mm and 29.4 mm downstream from the tracer inlet point in the
porous channel at Darcy velocity 0.22 mm/s. The overlaid profiles in each image indicate the fluorescent intensity variation across the channel between two horizontal rows of simulated soil grains................................................................. 57

Figure 3.4: Markers represent FITC-dextran normalized intensity profiles in MFD-I at a cross-section 29.2 mm downstream from the its inlet in experiments with motile bacteria (left plot), immobilized bacteria (middle plot), and no bacteria (right plot). Each marker represents pixel intensity averaged over ten pixels and the curves represent corresponding modeled profiles for the respective effective transverse dispersion coefficients obtained as the global average of triplicate experiments................................................................. 59

Figure 3.5: Average transverse dispersion coefficient (D_{xy}) values at different cross-sections of the device, estimated based on the average of three best fit model values for three experimental profiles at each cross-section in mixing experiments with motile bacteria. The x-axis represents longitudinal distance of the cross-sections from the inlet of FITC-Dextran into the porous bottom channel. Error bars indicate standard error from triplicate experiments.......................................................................................................................... 60

Figure 3.6: Variation of effective transverse dispersion coefficient, (D_{xy})_{eff}, with Darcy velocity in MFD-I under three different experimental conditions. Error bars represent standard error values of nine best fit profiles at three cross-sections of the device in triplicate experiments. The line represents results of the dispersion model (Eqn 3.6) with the best fit model parameter values given in table 3.1.............................................................................................................. 64

Figure 3.7: Effect of flow velocity on mixing enhancement due to bacterial random motility in different MFDs. Error bars indicate standard errors.................................................................................................................. 69

Figure 4.1: Schematic showing the details of the pore arrangement and sampling (imaging) area in the microfluidic device (MFD-IV). (a) The transverse pore arrangement simulates a two dimensional porous medium in which a low permeability layer of aquifer is sandwiched between two high permeability layers. (b) A zoomed-in view of the pore geometries of the high permeability zone (repeated pattern of uniform size grains) and low permeability zone (repeated pattern of two different sized grains). Also shown are the transverse pore throat areas where images were captured. (c) A representative microscopic image in the transverse pore throat with bacterial counting area highlighted in the middle of the pore throat. The transverse length of the counting area is 0.10 mm. Other properties of the device are given in the Table 4.1 and complete details of the overall physical geometry and the operation of the device are discussed in the previous chapter 3, section 3.3.1......................................................... 78

Figure 4.2: Results from Plug assay experiment to determine the optimal chemotaxtractant concentration of L-aspartic acid for Escherichia coli HCB33. Images in the upper row correspond to a representative chemotactic experiment (0.20 mM aspartic acid as chemotaxtractant in plug) and lower row images show results from a representative control experiment (no chemotaxtractant in plug) at various time intervals. The horizontal arrow in each image indicates the cross-section along which the grey intensity is analyzed and is accompanied by the intensity profile that represents bacterial concentration distribution surrounding the plug................................................................................................................. 83

Figure 4.3: Representative relative bacterial count profiles in chemotactic (CHTX) and non-chemotactic (CTRL) experiments along with normalized attractant concentration profiles (ATTR, in chemotactic experiments only) in transverse pore throats in experiments at Darcy
velocity 0.11 mm/s. Bacterial counts in pore throats at cross-sections 10 mm and 20 mm are relative to the bacterial count in the corresponding pore throats at a base cross-section 5 mm downstream from the attractant/buffer inlet into the porous channel. Attractant concentration profiles were normalized with respect to the maximum concentration................................................................. 85

Figure 5.1: Live-dead bacterial plug assay experiment showing the chemotactic response of Pseudomonas putida F1 (P. putida F1) to trichloroethylene (TCE) diffusing from an agarose plug (row 1) and the toxic effect of high TCE concentrations on P. putida F1 (row 3) at 1, 5, 10, and 20 min. after bacterial introduction into the chamber. Dark semicircular regions on the left side of each image (rows 1 and 3) represent plugs that contain 13% (v/v) TCE, that diffuses out into the surrounding aqueous suspensions of stained (live, in row 1, and dead/damaged, in row 3) bacteria. Rows 2 and 4 illustrate normalized live bacterial concentration- and dead/damaged cell concentration- profiles of images in Rows 1 and 3, respectively; where the horizontal axis in each panel represents distance from the plug edge (number of pixels).................................................................................................................................................. 101

Figure 5.2: Green- (representing live cells at (a) t=0, (b) t=1h and (c) t=2.5h) and red- (representing dead cells at (d) t=0h, (e) t=1h and (f) t=2.5h) stained Pseudomonas putida F1 cells during a typical trichloroethylene toxicity assay........................................................................................................................................................................................................ 103

Figure 5.3: Survival ratios (S = N/No) of Pseudomonas putida F1 in trichloroethylene- (a) and toluene- (b) stressed samples (observed (◇), linear model (—) and exponential model (—)) and non-stressed samples ( ) at different time intervals throughout toxicity experiments. Error bars represent standard errors for five replicate experiments............................................................................................................................. 105

Figure 5.4: Effect of staining and different trichloroethylene (TCE) exposure durations on growth curves of Pseudomonas putida F1 measured in terms of OD590. Blank control (◇) culture was neither stained nor exposed to TCE, ‘unstressed control’ (□) was stained but not exposed to TCE, dead control ( ) was resuspended in 70% isopropyl alcohol and remaining cultures were exposed to TCE for 1 hr (O) and 2.5 hrs (Δ).............................................................................................................................................................. 106

Figure 5.5: Observed survival ratio (S = N/No) of Pseudomonas putida F1 with 1% (v/v) trichloroethylene (TCE) - ( △ ) and toluene- (○) stressed samples. (—) and (—) represent best fit model prediction values for TCE (exponential) and toluene (linear), respectively. Error bars represent standard error in the survival ratio for triplicate experiments......................................................................................................................... 111

Figure 6.1: Contaminant removal from low permeability region of a heterogeneous porous medium. Non-chemotactic contaminant degrading bacteria flowing with groundwater remain dispersed across the aquifer (A) while chemotactic bacteria respond to the chemical gradient formed as a result of contaminant diffusion out of the low permeability region and accumulate around the low permeability region (B). This bacterial accumulation around the low permeability region may enhance the contaminant consumption rate which can result in even steeper gradient and faster dissolution of contaminant as a result................................................................. 115

Figure 6.2: Chemotaxis assisted bioaugmentation (injecting microbial cultures into aquifer) and/or biostimulation (adding nutrients to stimulate growth of existing subsurface microorganisms) strategy for an aquifer contaminated with leaking underground storage tank. Panel on the left (A) represents initial bacterial/nutrient addition to a contaminated site. Non-chemotactic bacteria remain dispersed in the contaminated reason and may wash away with flowing groundwater (B). However, chemotactic bacteria respond to the gradients
created as a result of contaminant consumption by them, and their ability to swim against and transverse to flow direction allow them to move toward higher concentration of contaminant in both longitudinal and transverse directions (C)………………………………………………………….. 117

Figure 7.1: Schematic showing various limiting factors in bioremediation that can be potentially enhanced due to bacterial motilites and their overall implication in improving bioremediation process…………………………………………………………………………………………………… 126

Figure A.1: Schematic representing the bi-layer micro-fluidic device (MFD) used for testing transverse chemotactic migration of bacteria in dual-permeability porous media. The device design is similar to that presented in Figure 3.1 except the pore arrangement was modified to represent dual-permeability region. Cross-sections representing sampling locations at 4.9, 9.8, and 19.6 mm downstream from the attractant inlet point into the porous channel are also shown. The other specific pore geometry details are given in Figure 4.1 and Table 4.1…… 145

Figure B.1: Comparison of concentration profiles of blank (no TCE) and TCE-containing plug assay experiments at different time intervals. Panels in rows 1 and 2 represent live cells (stained green) in blank and TCE-containing samples, respectively. The change in normalized concentration profile patterns of live cells (row 2) over the course of an experiment with TCE-containing samples in contrast with the nearly uniform live cell concentration profiles (row 1) in blank samples suggests a chemotactic response of P. putida F1 toward TCE. Toxicity effects of TCE can be seen by comparing row 3 (blank sample) and row 4 (TCE-containing plug). TCE diffusion into the bacterial chamber induces an accumulation of dead cells (stained red) over the course of an experiment, and is evident row 4, but not in row 3. For the purpose of comparison, concentration profiles are plotted on similar scales for both blank (row 1) and TCE-containing (row 2) samples for live cells and similarly for the dead cells (rows 3 &4)…… 146

Figure C.1: pH dependence of DL- aspartic acid concentration. Error bars indicates standard deviation of triplicate experiments………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………...
ABSTRACT

Bacterial Enhancement of Contaminant Bioavailability: Effects of Random Motility and Chemotaxis
Rajveer Singh
Supervisor: Dr Mira S. Olson

Understanding bacterial and contaminant transport in subsurface porous media is critical both in preventing contamination of drinking-water supplies and in successful implementation of bioremediation. Groundwater bioremediation is often limited by inadequate distribution of bacteria within the contaminated sites, which reduces the bioavailability of contaminants. Bacterial motility mechanisms, random motility and chemotaxis (directed movement toward or away from chemicals), can potentially help improve these limitations, thereby enhancing in situ bioremediation.

Microfluidic devices (MFDs) were designed and fabricated to simulate two-dimensional groundwater aquifer contamination scenarios. Three major studies were performed. First, the effect of bacterial random motility on contaminant mixing was studied in three different pore structure designs at four different flow rates. In uniform grain size with large pore throats (MFD-I) and non uniform grain size with restricted pore space (MFD-II) devices, the motile bacteria contributed to a nearly 2.3-fold and 3-fold increase in measured apparent transverse dispersivity ($\alpha_{app}$), respectively. No appreciable change was observed in a uniform grain size device with smaller pore throats (MFD-III).

In the second study, bacterial (Escherichia coli (E. coli) HCB33) chemotactic migration (in response to the chemoattractant DL-aspartic acid) toward a low permeability region of dual-permeability MFD (MFD-IV) was studied. Chemotaxis significantly increased (1.09 to 1.74 times)
observed bacterial counts in the low permeability region of the MFD. An accumulation of chemotactic bacteria was observed at the interfaces between the two permeability regions.

The third study focused on evaluating viable bacterial (*Pseudomonas putida* F1 (*P. putida* F1)) distribution in chemical gradients and the toxic effects of prolonged exposure to high concentrations of model contaminants (trichloroethylene (TCE) and toluene). Viable bacteria were observed to move away from high concentration of TCE. Toxicity of TCE and toluene to *P. putida* F1 were best described by exponential and linear viability-decay models, respectively, with viability-decay constants $k_{\text{TCE}} = 0.025 \text{ h}^{-4.95}$ ($r^2 = 0.965$) and $k_{\text{toluene}} = 0.198 \text{ h}^{-1}$ ($r^2 = 0.972$).

This study presents the first attempt to incorporate mixing due to bacterial motility in contaminant transport as well as the first experimental demonstration of bacterial chemotactic migration toward a contaminant source trapped in a low-permeability region. Results obtained will advance current understanding of the processes that may help improve mass transfer limitations of bioremediation strategies for contaminated sites.
CHAPTER 1: INTRODUCTION

Ground water is an invaluable resource on the earth. Over half of the United States population relies on ground water as a source of drinking water (USEPA 1999). The use of recalcitrant pollutants in industrial applications and their release into effluent waters over the years has resulted in contamination of a large number of groundwater aquifers and is therefore a major threat to drinking-water supplies around the world. According to a report by The US Environmental Protection Agency (EPA), there are a total 294,000 contaminated sites within the United States alone scheduled to be remediated between 2004-2033 under different programs, with an estimated treatment cost of approximately $209 billion (USEPA 2004).

1.1. Motivation

Contaminated ground water is difficult to characterize and remediate, often requiring decades of treatment and monitoring (USEPA 1999). Recognizing the prohibitively high cost and impracticality of fully removing all trapped contaminant sources (USEPA 1993), researches have explored in situ bioremediation and biological stabilization as safe and efficient alternatives to physicochemical remediation strategies (Herbes and Schwall 1978; Pandey and Jain 2002). Bioremediation refers to mineralization and/or transformation of pollutants into less harmful compounds via microbial degradation (Bedient et al. 1997). In addition to the several smaller-scale applications of bioremediation, shore-line clean-up efforts in Prince William Sound, Alaska, following the Exxon Valdez oil spill of 1989, is one example of a major application of bioremediation (Boopathy 2000).

Bioremediation requires an understanding of the complex multiphase subsurface environment, which includes microorganisms, contaminants, the soil matrix and its characteristics, and other factors such as nutrients and physicochemical conditions. Therefore,
to achieve successful bioremediation of a contaminated site one requires a well coordinated interdisciplinary approach among various disciplines such as microbiology, biochemistry, engineering, hydrogeology, soil science, and ecology (Boopathy 2000). Even though the application of the bioremediation at various sites has been demonstrated for reducing pollutant concentrations, there are several issues that remain to be addressed in order to employ bioremediation as a comprehensive technology to achieve complete treatment of contaminated aquifers. Some of the most important scientific factors that hinder the bioremediation process are: (1) Biodegradability of contaminant- whether the contaminant can serve as a source of energy for microorganisms directly or indirectly, (2) Bioavailability- rate of contaminant mass transfer (usually diffusion) to the microbial cells, and (3) Bioactivity- the operating state of microbial degradation processes (Boopathy 2000). This research work focuses on understanding processes that may help improving bioavailability of contaminants as well as bioactivity of microorganisms.

In order to overcome the aforementioned limitations to bioremediation, advective and dispersive transport of bacteria and contaminants in the subsurface, and characterization of biochemical reactions involved in pollutant biodegradation have been major areas of research in the past couple of decades. The roles of enzymes and genes in biodegradation are now relatively well understood. Current research efforts are focused on identifying additional features of bacterial behavior that may help enhance the rate of bioremediation (Parales and Harwood 2002). Bacterial motility mechanisms, including random motility and chemotaxis (a directed movement toward or away from chemicals (energy sources)), are two such bacterial behaviors that can potentially enhance in situ bioremediation, if properly harnessed. The motility of bacteria in aqueous media induces chaotic movement in their micro-surroundings which may enhance contaminant mixing and therefore may be helpful in overcoming mass transfer
limitations. Chemotaxis on the other hand, may enable bacteria to locate, penetrate, and remediate pollutant sources. Additionally, high concentrations of some environmental pollutants may be toxic to bacteria despite the fact that they are degradable at lower concentrations. Chemotaxis may help bacteria to move away from toxic concentrations of pollutants and instead accumulate in areas that are better suited for the biodegradation of pollutants.

The work contained in this thesis focuses on understanding the impact of bacterial motility mechanisms, random motility and chemotaxis, on bioremediation of contaminated aquifers. The findings of this research are helpful in understanding micro-scale processes in groundwater aquifers, and may significantly contribute to improving upon some of the limitations to successful in situ bioremediation of aquifers. We have demonstrated a significant increase in contaminant mixing due to bacterial random motility, and have also verified bacterial migration toward a chemoattractant (contaminant surrogate) in low permeability regions; both of these mechanisms may help in improving the bioavailability of contaminants. We have also developed contaminant toxicity prediction models for model contaminants (trichloroethylene and toluene) that can assist in designing improved remediation strategies for contaminated sites.

1.2. Research objectives

For successful bioremediation of a contaminated site, it is critically important to have the right bacteria in the right place under the right environmental conditions. The central focus of this research is to investigate if bacterial motility mechanisms, random motility and chemotaxis, can help overcome limitations in contaminant bioavailability in order to improve overall bioremediation potential. The specific objectives are as follows:
1) To assess enhancement in contaminant (fluorescein isothiocyanate (FITC) labeled dextran as surrogate) mixing in porous media due to bacterial (Escherichia coli (E. coli) HCB33) random motility and to evaluate the effect of porous media geometry on the mixing enhancement using physically simulated two-dimensional porous microfluidic devices (MFDs).

2) To evaluate E. coli HCB33 chemotaxis in a dual-permeability heterogeneous porous medium using two-dimensional porous microfluidic devices (MFDs) and to quantify bacterial accumulation in low permeability regions containing a contaminant source.

3) To study kinetics of model contaminants’ (trichloroethylene and toluene) toxicity to contaminant-degrading bacteria (Pseudomonas putida (P. putida) F1) and to develop toxicity prediction models.

1.3. Dissertation structure

This dissertation is divided into seven chapters. The content of each chapter is as follows:

**Chapter 1:** This chapter covers general introduction and motivation behind this research work along with specific objects of the research and structure of the dissertation.

**Chapter 2:** This chapter presents background information that is helpful in understanding the research presented in subsequent chapters.

**Chapters 3-5:** These chapters contain the main research carried out during this study. Each chapter contains specific background information, detailed experimental procedures, data collection, and analyses. Results and discussion of contaminant mixing enhancement due to random motility, bacterial chemotaxis in dual permeability regions, and kinetics of contaminant toxicity to contaminant-degrading-bacteria are presented in chapter 3, chapter 4, and chapter 5, respectively.
Chapter 6: This chapter provides recommendations on how chemotaxis may be exploited to improve field-scale applications of *in situ* groundwater bioremediation.

Chapter 7: This chapter highlights important findings from this research and makes recommendations for future research topics.
CHAPTER 2: BACKGROUND

2. Introduction

This chapter presents the current-state-of-knowledge on the various research topics covered in this thesis. The first part of this chapter covers details of bacterial motility mechanisms - random motility and chemotaxis - their fundamental basis, measurement techniques, impact on overall bacterial transport, and significance in bioremediation of contaminated groundwater aquifers. The second part of this chapter presents a review of the basics of microfluidics and its application in chemical biology and environmental engineering, particularly with respect to groundwater remediation.

2.1. Bacterial transport in soil

Understanding microbial transport through subsurface porous media is critical both for characterizing contamination of drinking-water bodies and designing successful bioremediation strategies for contaminated sites. Various complex physicochemical and biological processes affect microbial transport in the subsurface, such as advection, dispersion, straining and filtration, size exclusion, and interaction between the microbial surfaces and soil grains (electrostatic and chemical processes) (Ginn et al. 2002). A good deal of research, including experimental, modeling and field-scale studies, has been devoted to understanding inorganic particle (usually colloid) transport, as a surrogate for microbes, in subsurface porous media, an extensive review of which is recently presented by Sen and Khilar (2006). Overall bacterial transport in groundwater aquifers is modeled using a conventional advection-dispersion equation which has been modified by several researchers to account for different porous media and bacterial characteristics. A simplified one-dimensional version of the equation modified for
interaction between bacteria and soil grains is given by the following equation (Scheibe et al. 2007).

\[
\frac{\partial B}{\partial t} = D \frac{\partial^2 B}{\partial x^2} - v_p \frac{\partial B}{\partial x} - K_f B + K_r \rho_b S \\
\frac{\partial S}{\partial t} = \frac{\varepsilon}{\rho_b} K_f C - K_r S
\]

where \( x \) is spatial location, \( t \) is time, \( B \) is suspended cell concentration (cells per volume water), \( S \) is attached cell concentration (cells per unit mass of sediment), \( v_p \) is average linear velocity, \( D \) is the dispersion coefficient, \( K_f \) is the bacterial attachment rate coefficient, \( K_r \) is the bacterial detachment rate coefficient, \( \rho_b \) is sediment bulk density, and \( \varepsilon \) is sediment porosity. The value of \( K_f \) is often given by colloid filtration theory as (Scheibe et al. 2007).

\[
K_f = \left[ \frac{3(1-\varepsilon)}{2d_g} \alpha \eta \right] v_f
\]

where, \( d_g \) is mean grain size of porous media, \( \alpha \) is collision frequency, and \( \eta \) is filtration efficiency.

However, using such models often results in inaccurate prediction of microbial transport which may be attributed to the influence of additional factors such as their interaction with chemicals/contaminants present in their surrounding, as well as biotic factors such as their growth and decay (Scheibe and Wood 2003). Bacterial motility mechanisms such as random motility and chemotaxis, explained in the following sections, may influence overall bacterial transport and are reported to play important roles in both the fate and transport of bacteria in subsurface porous media (Pandey et al. 2002; Parales and Harwood 2002; Olson et al. 2004; Hilpert 2005; Olson et al. 2006; Ford and Harvey 2007; Long and Hilpert 2007; Lanning et al. 2008; Singh and Olson 2008; Long and Ford 2009; Singh and Olson 2010). Therefore, a careful
consideration and appropriate incorporation of these factors in the advection-dispersion bacterial transport equation is important for accurate prediction of microbial transport in the subsurface. Successful prediction overall bioremediation relies on the ability of a model to accurately predict bacterial transport and to fully account for the various interactions among bacteria, pollutants, and porous media (Mulligan and Yong 2006).

2.1.1. Bacterial migration mechanisms

Among the several migration mechanisms that bacteria may pose in aqueous media, random motility and chemotaxis are critical in influencing overall bacterial transport and thereby bioremediation of contaminated aquifers. The following sections provide details on these bacterial migration processes.

2.1.1.1. Bacterial random motility

In a uniform environment, i.e. no stimuli, bacterial migration in aqueous media is random. A typical bacterial migration pattern, for example *Escherchia coli* (E. coli.), consists of relatively straight swimming runs, interrupted by tumbling events that change the swimming direction (Figure 2.1A). This alternating series of runs and tumbles is governed by the rotating 6-8 helical shaped flagella embedded in the cell membrane via a molecular motor. The counterclockwise rotation of the molecular motors results in the formation of a bundle of flagella behind the cell body that propels the bacteria in the forward direction. When one of the motor changes its direction to clockwise the flagellar bundle unravels and the cell tumbles chaotically and reorients itself for another run (Turner et al. 2000). This alternate run and tumbling movement of bacteria results in a 3D random walk that is somewhat analogous to the diffusion of gas molecules (Figure 2.1B) (Ford and Harvey 2007).
Figure 2.1: A typical bacterial motility mechanism consists of a series of straight runs and tumbling movements (A). The random motion of the bacteria results in chaotic movement where overall motion looks like a 3D-random walk similar to the diffusion of gaseous molecules (B). Under the influence of chemical gradients bacteria bias their random motion, either toward or away from the source, and the resulting motion looks like a biased random walk (C).
2.1.1.2. Bacterial chemotaxis

In the presence of chemical gradients in the surrounding medium, bacteria lengthen/shorten their run lengths between two consecutive tumbling movements; in other words, they change their tumbling frequency depending on the presence of chemical stimuli. This ability of bacteria to sense and respond to chemical gradients in their surroundings and to direct their migration either toward or away from increasing concentrations of chemicals is known as chemotaxis (Pandey and Jain 2002; Ford and Harvey 2007). Bacterial migration toward and away from chemicals helps bacteria navigate to niches that are optimal for their growth and survival (Pandey and Jain 2002) and is termed positive and negative chemotaxis, respectively. Generally, chemicals that attract bacteria as sources of carbon and/or energy are termed chemoattractants for those specific bacteria. Similarly, chemorepellents repel certain bacteria and are often toxic (Pandey and Jain 2002). Recently, it has been reported that chemical contaminants that are readily degraded at low concentrations are toxic to bacteria above certain concentration thresholds (Collins and Daugulis 1999; Kim et al. 2005). In such cases a chemical can act both as a chemoattractant (at low concentrations) and a chemorepellent (at high concentrations) for the same bacterial strain.

2.1.2. Why microorganisms pose chemotaxis

Responding to changes in the environment is a fundamental property of living cells and is of prime importance to single cell microorganisms as they interact with their changing environment (Pandey and Jain 2002). Through evolution, microorganisms have developed mechanisms that help them regulate their cellular mechanisms to changing environments (Hoch 2000). Chemotaxis is an advantageous behavior selected by bacteria that has probably evolved as a result of bacterial pursuit of energy sources. It has been observed that numerous bacteria develop the ability to respond chemotactically to certain chemicals when they are grown under
specific carbon and energy source conditions. Childers et al. (Childers et al. 2002) reported that growth of flagella and pili, appendages responsible for chemotactic movement of bacteria, takes place on the surface of Geobacter metallireducens cells only when cells are grown under insoluble electron acceptor conditions. In contrast, cells grown in the presence of soluble substrates are neither chemotactic nor do they develop flagella and pili. They concluded that chemotaxis enables bacteria to establish contact with insoluble electron acceptors. Pandey and Jain (Pandey and Jain 2002) suggest that bacteria developed taxis, including chemotaxis, aerotaxis and phototaxis, over time as naturally available energy sources became limited. Introduction of anthropogenic pollutants into the environment, along with increased competition for natural resources, may have given bacteria the ability to respond to and degrade these chemical pollutants as sources of carbon and energy. Figure 2.2 shows a schematic postulating the evolution of bacterial chemotaxis and other behavioral responses on a geological time scale put forth by Pandey and Jain (2002).

2.1.3. Molecular mechanisms of chemotaxis

Cell communication with their environment consists of complex extracellular signal reception and intracellular signal transduction mechanisms. A simple mechanism for Escherichia coli (E. coli) chemotaxis toward amino acids and sugars is described by Parales and Harwood (Parales and Harwood 2002). E. coli are able to sense and respond to changes in their environment through surface receptor molecules, called methyl-accepting chemotaxis proteins (MCPs) (Ford and Harvey 2007). These MCPs embedded in the plasma membrane usually bind the attractant molecules. Upon binding, this activates intracellular signaling proteins which in turn alter the function of the effector (Alberts 2002). This signal transudation mechanism results in a change in the direction of the flagellar rotation and is manifested as chemotaxis (Parales and Harwood 2002). The chemotactic machinery of E. coli consists of five MCPs and six chemotactic proteins
Complex signaling systems are found in many other chemotactic species with multiple sets of chemotactic genes. For example, 25 or more MCPs are found in *Pseudomonas putida* (*P. putida*) (Parales and Harwood 2002). It is argued that despite these complexities, the fundamental mechanism of signal reception and transduction is similar in all species (Armitage and Schmitt 1997).

### 2.1.4. Random motility and chemotaxis assays

A number of assays have been developed to evaluate the role of random motility and chemotaxis in bacterial transport. These assays can be divided into two general groups: single-cell, and population-scale studies. A comparative study of bacterial random motility and chemotaxis quantification assays is presented by Lewus and Ford (Lewus and Ford 2001). In this section we describe experimental techniques for measuring and quantifying bacterial random motility and chemotaxis to specific chemoattractants before the advent of microfluidics.

#### 2.1.4.1. Capillary assay

This method was first introduced by Adler (Adler 1966, 1973) and has since been repeatedly modified to enable quantitative evaluation of chemotaxis. A typical capillary assay consists of a microcapillary tube filled with attractant and placed in a pool of motile bacteria at one end and sealed at the other (Figure 3A). Motile bacteria respond to the chemical gradient formed as a result of diffusion into the pool and swim up-gradient into the tube. The tube is removed after a pre-specified time interval and cells accumulated inside the tube are enumerated to quantify chemotaxis. A cloud of bacterial accumulation can also be observed microscopically around the mouth of the capillary for qualitative observation. Random motility can be quantified using similar experiments without a chemical attractant. Mathematical expressions for deriving quantitative expressions of random motility and chemotaxis from capillary assay data will be described in Section 2.1.5.1.
Figure 2.2: Schematic representing a possible evolution in the bacterial chemotactic response to environmental pollutants as a result bacterial pursuit for carbon and energy resources. Improved understanding of this acquired behavioral response of degradative bacteria can help to design enhanced in-situ bioremediation strategies.

* This figure is adopted from Singh and Olson (2008) with appropriate modification.
Figure 2.3: Bacterial chemotaxis assays. Swarm plate assay (panel A): A growth medium with low agar content is poured into a Petri dish. Motile bacteria are stabbed in the middle. A sharp chemotactic band moving outward from inoculation point can be observed (A(i)) in as comparison to blank (A(ii)). Drop Assay (panel B): Similar to swarm plate assay except bacteria are suspended in drop assay medium and attractant is placed at the centre of Petri dish. Chemotactic bacterial band can be observed around the attractant (B(ii)) in comparison to control (B(i)). Capillary assay (panel C): Attractant, placed in capillary tube diffuses out in pool of motile bacteria. In response to chemical gradient cells accumulate at the mouth of capillary which can be observed under microscope. Agarose plug assay (panel D): attractant is mixed with melted agarose plug in the middle of chamber formed by cover slip and plastic strips; and bacterial suspension is flooded into chamber. Chemotactic band can be visualized under microscope.

*Note: This Figure is adopted from our publication Singh and Olson (2008)*
Capillary assays are relatively easy to perform and have been widely used for quantitative evaluation of chemotaxis. However, data obtained from this method is not useful for relating population behavior to intrinsic cell parameters (Ford et al. 1991). Mathematical models account for variability in setup geometry, but since flow patterns at the mouth of the capillary are complex, such models are based on simplified boundary conditions. This method is particularly suitable for testing chemotaxis of sparingly soluble compounds, such as naphthalene (Parales and Harwood 2002).

2.1.4.2. Stopped-flow diffusion chamber (SFDC) assay

Figure 3B represents an experimental design for the stopped-flow diffusion chamber (SFDC) introduced by Ford et al. (Ford et al. 1991) for quantification of bacterial random motility and chemotaxis. This assay is based on the principle that two liquid streams, with different chemoattractant or chemorepellent concentrations, intersect via impinging flow to form a step change in chemical concentration. Chemotactic bacteria respond either positively or negatively to the chemical gradient. This assay overcomes limitations of the capillary assay by providing a well characterized chemical concentration gradient and boundary condition.

Briefly, bacteria that are uniformly distributed in the SFDC respond to a chemical gradient induced by an initial step change in attractant concentration and migrate toward optimal concentrations, resulting in a moving bacterial band as time progresses (Figure 3B). Light scattering microscopy is used to detect moving bacterial bands. For random motility quantification, similar experiments can be performed with no chemoattractant.

Temporal bacterial concentration profiles can be obtained from digitized images and transformed into dimensionless bacterial concentration versus position plots (Figure 3B). The area under the crest or above the trough represents bacterial concentration and is proportional to the number of migrated bacteria. Ford et al. (Ford et al. 1991) show that the area above the
trough is linearly proportional to the square root of time. The following expression can be used to determine the random motility coefficient (Lewus and Ford 2001):

\[ \frac{b_0}{2} \sqrt{\frac{4\mu_0 t}{\pi}} = A_{\text{exp}} \]

where, \( b_0 \) is initial bacterial concentration in the injected solution; \( \mu_0 \) is the random motility coefficient; \( t \) is elapsed time; and \( A_{\text{exp}} \) is the experimentally determined area under the bacterial concentration vs position curve. The random motility coefficient can be calculated using slope of the \( A_{\text{exp}}/b_0 \) vs \( t \) curve and Eq. 2.4. A detailed procedure for determining chemotaxis parameters is provided by Lewus and Ford (Lewus and Ford 2001). One of the main advantages of using this method is that it provides well-defined boundary conditions for attractant and bacterial concentrations in both spatial and temporal coordinates, and ease in mathematical analysis of experimentally obtained data. In addition, the initial chemical concentration is easily adjusted, enabling controlled studies of the chemotactic response at varying contaminant concentrations.

The SFDC has been used to confirm that \( P. \ putida \ F1 \) exhibits both positive and negative chemotaxis toward benzene at low and high chemical concentrations, respectively.

### 2.1.4.3. Agarose plug assay

The agarose-in-plug bridge method was first developed by Yu and Alam (Yu and Alam 1997) for studying chemotaxis. In this method, an attractant or repellent is mixed with low-melting temperature agarose and a drop of mixture is placed on the top of a microscope slide. A cover slip supported by plastic strips at both ends is placed on top to form a chamber around the agarose plug (Figure 3C). A bacterial suspension is flooded into the chamber around the plug. A characteristic chemotactic band of bacterial accumulation is visualized surrounding the plug a small distance from its edge using light scattering microscopy. The limitation of this method is its poorly defined boundary conditions due to variability in the shape of the plug, making it difficult
to model mathematically. This method has been used for toluene (Parales et al. 2000) and TCE (Parales et al. 2000; Olson et al. 2004), and is particularly useful for volatile compounds, as the system is partially closed and therefore minimizes volatilization losses of the chemical (Parales and Harwood 2002).

2.1.4.4. Swarm plate assay

This qualitative method is based on metabolism of an attractant. A carbon source (attractant) is mixed with low percentage agar media (generally 0.3 % (Parales and Harwood 2002)) and poured in a petri dish. Bacteria are stabbed in the middle of the petri dish and incubated. A sharp chemotactic band of bacteria growing outward is visualized in the petri dish as a result of attractant metabolization and the resultant concentration gradient (Figure 3D). This method is well suited for identification of chemotactic bacteria and is widely used for enrichment of chemotactic mutants (Harwood et al. 1994; Bhushan et al. 2000; Samanta et al. 2000; Pandey et al. 2002; Parales and Harwood 2002). However, as metabolism is a primary requirement, this method is limited to testing chemotaxis of metabolizable chemoattractants (Parales and Harwood 2002).

2.1.4.5. Drop assay

Similar to the swarm plate assay, this method is also based on population-scale imaging and may be used to study bacterial responses to both chemoattractants and chemorepellents. Chemotactic bacteria are suspended in a drop assay agar consisting of bacto-agar (0.3 %) and a carbon source (1 mM glucose, for example) (Samanta et al. 2000), and poured into a petri dish. The chemotactic response is determined by placing an attractant/repellent in the center of the petri dish, incubating, and observing the characteristic chemotactic band formed surrounding the drop.
2.1.4.6. Three dimensional tracking microscopy

In addition to the population-based assays described above, individual cell tracking assays for evaluating random motility and chemotaxis are also described in the literature (Lovely and Dahlquist 1975; Ford et al. 1991; Lewus and Ford 2001), and are based on a three-dimensional tracking microscope developed by Berg (Berg 1971). This method consists of characterizing the 3-D random walk, described earlier in section 2.2, of individual bacteria (Figure 3E). The parameters of interest are average run length ($\lambda$), average run time ($t$), and average turn angle ($\alpha$) of the bacteria. The following mathematical relationship, derived by Lovely and Dahlquist (Lovely and Dahlquist 1975) and based on statistical analysis of individual cell based parameters, can be used to calculate a population-based random motility coefficient.

$$\mu_0 = \frac{\lambda v_c}{3} \frac{1}{1 - \cos \alpha}$$

where, $v_c$ is the average chemotactic velocity and can be calculated from the average run length and average run time.

2.1.4.7. Chemotactic index (C.I.)

A chemotaxis index parameter has been reported (Pandey et al. 2002) to compare the chemotactic response of different species to the same attractant. In a capillary assay experiment, C. I. is defined as the ratio of the number of cells accumulated in the capillary with attractant to that in the control.

2.1.5. Chemotaxis transport parameters

To evaluate the potential applicability of chemotaxis to in situ bioremediation of environmental pollutants in soil and ground water, it is important to quantify this process in terms of quantifiable chemotactic transport parameters that can be directly incorporated into comprehensive bacterial fate and transport models. Ford and Harvey (Ford and Harvey 2007) have recently presented a systematic approach for quantification of these response parameters.
for laboratory-scale studies. This section presents a review of the transport parameters commonly reported in the literature.

### 2.1.5.1. Chemotactic sensitivity coefficient ($\chi_0$)

The chemotactic sensitivity coefficient, $\chi_0$, is an intrinsic cell population based parameter that theoretically relates the individual swimming behavior of a bacterium to the resulting migration of the bacterial population (Ford and Lauffenburger 1992). This parameter accounts for the mechanism by which bacteria respond to chemical gradients. Ford and Lauffenburger (Ford and Lauffenburger 1992) derived the following analytical expression for determining the chemotactic sensitivity coefficient for commonly used capillary assay experiments:

$$\chi_0 = \sqrt{D\mu_0 \left(1 + \frac{C_0}{C_\infty} - \frac{N}{N_{RM}} - 1\right)}$$  \hspace{1cm} 2.6$$

where $D$ is attractant diffusivity; $C_0$ and $C_\infty$ are normalized attractant concentrations at the mouth and far end of the capillary, respectively. Concentrations are normalized by the chemotaxis receptor constant, $K_d$, (defined later); $N$ and $N_{RM}$ are numbers of cells accumulated in the capillary in the presence and absence of chemoattractant, respectively; and $\mu_0$ is the cell random motility coefficient derived by Segel et al. (Segel et al. 1977) :

$$\mu_0 = \frac{\pi}{4t} \left(\frac{N_{RM}}{N_{RM}^2 r^2 b_c}\right)^2$$  \hspace{1cm} 2.7$$

where $N_{RM}$ is the number of cells accumulated in a capillary of radius $r$ at time $t$; and $b_c$ is the initial bacterial cell concentration in the chamber.
2.1.5.2. Chemotaxis receptor constant ($K_d$)

Chemoreceptor half saturation constant and chemoreceptor binding constant are terms that are used interchangeably in the literature for the chemotaxis receptor constant, $K_d$. This parameter represents the propensity of bacteria to bind the attractant and characterizes the binding between attractant molecule and cell surface receptors, which are responsible for sensing concentration gradients in the surroundings. The value of this parameter corresponds to an optimal chemoattractant concentration, where bacteria elicit the strongest chemotactic response, and hence has units of concentrations (e.g. mM). This parameter is generally determined from dose-response curves, but can also be determined as a best fitted model parameter (Marx and Aitken 1999; Olson et al. 2004).

2.1.5.3. Chemotactic velocity ($v_c$)

The chemotactic velocity, $v_c$, is defined as an advective transport property and is a function of bacterial species, chemoattractant concentration, and chemoattractant concentration gradient. The following mathematical expression is commonly used for describing chemotactic velocity in shallow gradients (Chen et al. 1998):

$$

v_c = \frac{\chi_0}{3} \frac{K_d}{(K_d + C)^2} \frac{dC}{dx}

$$

for a one dimensional system, where $C$ is chemoattractant concentration; and $x$ is the spatial coordinate. There may exist a critical chemoattractant concentration, $C_c$, below which no chemotactic response is elicited. Considering this fact, we suggest the following mathematical expression could be useful in calculating chemotactic velocity at low attractant concentrations:

$$

v_c = \Theta(C - C_c) \frac{\chi_0}{3} \frac{K_d}{(K_d + C)^2} \frac{dC}{dx}

$$

where $\Theta(C)$ is a Heaviside step function the value of which is given by,
Table 2.1: Random motility and chemotactic sensitivity coefficients of different bacterial strains under different experimental conditions.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Chemoattractant</th>
<th>Random Motility Coefficient (x 10^6 cm^2/sec)</th>
<th>Chemotactic sensitivity coefficient (x 10^4 cm^2/s)</th>
<th>Experimental condition</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. putida</em> F1</td>
<td>TCE</td>
<td>0.15</td>
<td>0.08</td>
<td>Glass coated polystyrene (250 µm dia)</td>
<td>Olson et al (2004)</td>
</tr>
<tr>
<td><em>P. putida</em> G7</td>
<td>Naphthalene</td>
<td>0.32</td>
<td>0.72±0.14 1.3</td>
<td>Bulk liquid saturated glass beads (50µm)</td>
<td>Marx and Aitken (1999) Pedit et al (2002)</td>
</tr>
<tr>
<td><em>P. putida</em> PR2000</td>
<td>3-chloro benzene</td>
<td>35±2 0.7 1.6 3.1</td>
<td>1.9±0.7</td>
<td>Bulk aqueous media quartz sand of dia 81 µm 194 µm 326 µm</td>
<td>Barton and Ford (1995)</td>
</tr>
<tr>
<td><em>E. coli</em> AW405</td>
<td>α-methasparate</td>
<td>2.9 ± 1.4 3.8 ± 0.2 2.5 ± 0.4</td>
<td>2.4 ± 0.6</td>
<td>Bulk liquid cell swimming speed SFDC method Capillary assay SFDC method Microchannel Plate pore channels</td>
<td>Lewus and Ford (2001) Strauss et al (1995) Berg and Turner (1990)</td>
</tr>
<tr>
<td><em>E. coli</em> NR50</td>
<td>Fucose</td>
<td>4.7±0.8</td>
<td>0.39±0.01 4.1</td>
<td>Bulk liquid cell swimming speed SFDC method Capillary assay SFDC method Microchannel Plate pore channels</td>
<td>Strauss et al (1995)</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>Fucose</td>
<td>11±4</td>
<td>0.8±0.3</td>
<td>Bulk liquid cell swimming speed SFDC method Capillary assay SFDC method Microchannel Plate pore channels</td>
<td>Ford et al (1991)</td>
</tr>
</tbody>
</table>

*Note: Diffusion coefficients values reported above may not necessarily measured under similar experimental conditions as for chemotactic and random motility coefficients.

**These values are reported by Ford and Harvey (2007) based on drift velocity values from Berg and Turner (1990).
The chemotactic velocity parameter is directly incorporated into bacterial transport models as an advective velocity term to account for bacterial transport due to chemotaxis.

2.1.6. Chemotactic bacterial transport models

Mathematical models describing bacterial transport are modified to account for chemotaxis in porous media. One such model given by Pedit et al. (2002) and Olson et al. (2006) is based on a material balance for bacteria and chemoattractant in one dimension and can be represented as:

\[
R \frac{\partial b}{\partial t} = - \frac{\partial (bv_c)}{\partial x} + D_b \frac{\partial^2 b}{\partial x^2} - \frac{\partial (bv_c)}{\partial x} + Y \frac{q_m C}{C + k_s} b - k_d b
\]

and,

\[
R \frac{\partial C}{\partial t} = - \frac{\partial (Cv_c)}{\partial x} + D_c \frac{\partial^2 C}{\partial x^2} - \frac{q_m C}{C + k_s} b
\]

where, \(b\) is bacterial concentration; \(C\) is chemoattractant concentration; \(R\) is the retardation factor; \(v_x\) is the advective ground water velocity, \(v_c\) is the chemotactic velocity; \(Y\) is the yield coefficient; \(q_m\) is the maximum chemoattractant utilization rate; \(k_s\) is the half saturation constant; \(k_d\) is the bacterial decay rate; \(t\) is time; and \(x\) is the spatial coordinate in the direction of ground-water flow. \(D_b\) and \(D_c\) represent the longitudinal bacterial and chemical dispersion coefficients, respectively, and are given by:

\[
D_b = \alpha v_x + \frac{\mu_{eff}}{\varepsilon}
\]

and

\[
D_c = \alpha v_x + \frac{D_{eff}}{\varepsilon}
\]

where \(\alpha\) is the longitudinal dispersivity; \(\varepsilon\) is porosity; \(\mu_{eff}\) is the effective bacterial motility coefficient in porous media; and \(D_{eff}\) is the effective diffusion coefficient of the chemoattractant.
2.1.7. Review of bacterial chemotaxis toward environmental pollutants

Various natural and anthropogenic activities have led to the expulsion of simple and polycyclic aromatic hydrocarbons (PAHs), nitroaromatic compounds (NACs) and chlorinated compounds into the environment (Pandey et al. 2002). Although the biodegradability of many of these compounds has been studied, bacterial chemotaxis toward environmental pollutants, which may be used to enhance remediation rates, has received relatively little attention (Pandey and Jain 2002). Recently, a number of soil inhabiting bacteria were found to be chemotactic toward a variety of environmental pollutants and have been extensively reviewed by Pandey and Jain (Pandey and Jain 2002) and Parales and Harwood (Parales and Harwood 2002). In several studies (Harwood et al. 1994; Parales et al. 2000; Samanta et al. 2000; Pandey and Jain 2002; Parales and Harwood 2002; Bhushan et al. 2004), a direct or indirect correlation between chemotaxis and biodegradation has been reported and it is speculated that chemotaxis may potentially enhance bioremediation of contaminated soil and ground water. A comprehensive list of environmental pollutants and the identified bacterial strains that exhibit chemotaxis toward them is presented in Table 2.2.

2.1.7.1. Chemotaxis toward simple aromatic compounds

A large number of simple aromatic compounds are environmental pollutants, including benzene and toluene. Petroleum products, asphalt, coal tar, creosote and incomplete combustion of fossil fuel are the major sources of these compounds (Bedient et al. 1997). As a result of human activities in the extraction, transportation, refinement and use of petroleum, these compounds have become serious environmental threats (Parales and Harwood 2002). Due to their simple structure, mineralization of these compounds is relatively easy and thereby favorable for biodegradation. A number of soil bacteria, *Pseudomonas* sp., *Ralstonia* sp., *Burkholderia* sp. and *Rhizobium* sp, for example, exhibit chemotaxis toward aromatic hydrocarbons (Table 2.2).
Chemotaxis can play an important role in biodegradation of these compounds by bringing degrading bacteria closer to sites that are contaminated with these pollutants.

2.1.7.2. Chemotaxis toward polycyclic aromatic compounds (PACs)

Polycyclic aromatic hydrocarbons are compounds that consist of two or more aromatic rings. Naphthalene, one of the most prevalent groundwater contaminants at sites contaminated with PACs (Durant et al. 1995), is relatively easily degraded and is often used as a model compound in degradation studies of PACs. A number of bacterial strains are chemotactic toward naphthalene, including *P. putida* G7, *Pseudomonas*. sp. strain NCIB 9816-4 and *P. putida* RK J1 (Table 2.2). The chemotactic responses in G7 and NCIB 9816-4 species were induced when grown with naphthalene itself (Grimm and Harwood 1997), whereas in RK J1 species, the response was induced by growing on salicylate (Samanta and Jain 2000). The naphthalene chemoreceptor, NahY (an MCP), is encoded downstream of the naphthalene catabolic genes on the NAH7 plasmid (Parales and Harwood 2002). Pandey and Jain (Pandey and Jain 2002) suggest that chemotaxis toward naphthalene and/or salicylate might be due to a change in cellular energy levels due to metabolism of these compounds and/or because of intracellular receptors that recognize such contaminants or their degradation intermediates.

2.1.7.3. Chemotaxis toward nitroaromatic compounds (NACs)

Nitroaromatic compounds are man-made pollutants and are difficult to degrade. They are generally used as pesticides, herbicidal dyes, and explosives. Once released into the environment, NACs undergo complex physical, chemical and biological changes, resulting in harmful and toxic byproducts (Samanta et al. 2000; Paul et al. 2006). Samanta et al. (Samanta et al. 2000; Paul et al. 2006) isolated Ralstonia sp SJ98 from pesticide contaminated soil using the drop assay technique. Strain SJ98 is chemotactic toward a large number of NACs and subsequently degrades them by metabolism (Bhushan et al. 2000; Samanta et al. 2000) and co-
Table 2.2: List of bacterial strains that are chemotactic toward various environmental pollutants.

<table>
<thead>
<tr>
<th>Pollutant Group</th>
<th>Bacterial Stain</th>
<th>Chemoattractants</th>
<th>Comments</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple or single ring aromatic hydrocarbon</td>
<td><em>Pseudomonas putida</em> PRS2000</td>
<td>Benzoate; m-Toluate; p-Toluate; o-Toluate; Salicylate; p-Hydroxybenzoate; Benzoylformate; β-Phenylpyruvate; Salicylate; DL-p-Hydroxymandelate; Phenoxyacetate</td>
<td>Metabolism</td>
<td>Harwood et al 1984</td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em> F1</td>
<td>Toluene; Benzene, Ethylbenzene; Succinate</td>
<td></td>
<td>Harwood (4)</td>
</tr>
<tr>
<td></td>
<td><em>Ralstonia pickettii</em> PKO1</td>
<td>Toluene</td>
<td></td>
<td>Harwood (4)</td>
</tr>
<tr>
<td></td>
<td><em>Burkholderia cepacia</em> G4</td>
<td>Toluene</td>
<td></td>
<td>Harwood (4)</td>
</tr>
<tr>
<td></td>
<td><em>Azospinillum</em> strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Rhizobium meliloti</em></td>
<td>Benzoate; catechol (1,2-dihydroxybenzene); 4-hydroxybenzoate; 3,4-dihydroxybenzoic acid; Malate; 4',7-dihydroxyflavone; 4',7-Dihydroxyflavanone; 4,4'-dihydroxy-2-methoxychalcone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas. sp.</em> strain NCIB 9816-4</td>
<td>Naphthalene; Salicylate</td>
<td></td>
<td>Grimm and Harwood 97</td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em> RK J1</td>
<td>Naphthalene, Salicylate</td>
<td></td>
<td>Samanta and Jain 2000</td>
</tr>
<tr>
<td>Nitroaromatic Compounds (NACs)</td>
<td>Ralstonia sp. SJ98</td>
<td>Ralstonia sp. SJ98</td>
<td>Clostridium sp. EDB2</td>
<td>Pentachlorophenol (PCP); 2,4-dichlorophenoxyacetate (2,4-D); 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); Hexachlorobenzene (HCB); p-nitrophenol (PNP); 4-nitrocatechol (NC); o-nitrobenzoate (ONB); p-nitrobenzoate (PNB); 3-methyl-4-nitrophenol (MNP); 2,4-dinitrophenol; 2,5-dinitrophenol; 2,6-dinitrophenol; o-dinitrobenzene; m-dinitrobenzene; m-nitrophenol.</td>
</tr>
<tr>
<td>Chlorinated Hydrocarbon</td>
<td><em>P. putida</em> PRS2000</td>
<td><em>P. putida</em> F1</td>
<td><em>Ralstonia eutropha</em> JMP123(pJP4)</td>
<td><em>Pseudomonas stutzeri</em> KC</td>
</tr>
</tbody>
</table>
metabolism (Pandey et al. 2002) (Table 2.2). Bhushan et al. (Bhushan et al. 2004) reported chemotaxis-mediated biodegradation of cyclic nitramine explosives by an obligate anaerobic bacterium Clostridium sp. strain EDB2 (Table 2.2), which they isolated from marine sediments.

On the contrary, a number of other compounds such as p-nitroaniline, 2,3-dinitrotoluene, naphthalene, phenanthrene and salicylic acid, were neither biodegradable by nor chemoattractants for strain SJ98 (Samanta et al. 2000). These results further reinforce the hypothesis that a correlation between chemotaxis and biodegradation exists.

2.1.7.4. Chemotaxis toward chlorinated compounds

Both aliphatic and aromatic chlorinated hydrocarbons are of growing concern in the environment due to their abundant use as herbicides, pesticides, and solvents in various industries (Harwood et al. 1990). Dichloroethylene (DCE), trichloroethylene (TCE) and perchloroethylene (PCE) are common pollutants in the environment and most of them are recalcitrant to biodegradation (Harwood et al. 1990)

Some bacterial strains that are chemotactic toward chlorinated compounds have been isolated for the biodegradation of chlorinated compounds (Table 2.2). A chemotactic response toward 2,4-dichlorophenoxyacetate (2,4-D) is induced in R. eutropha JMP123(pJP4) when grown on 2,4-D, which encodes the pJP4 plasmid containing genes for 2,4-D degradation (Pandey and Jain 2002). P. putida PRS2000 exhibits chemotaxis to 3- and 4-chlorobenzoate, when induced with 4-hydroxybenzoate (Harwood 1989; Harwood et al. 1990). P. putida F1, grown on toluene, is chemotactic toward trichloroethylene (TCE), dichloroethylene (DCE), perchloroethylene (PCE), and other chlorinated compounds (Parales et al. 2000).

A review of the molecular basis of chemotaxis toward different pollutants indicates that chemoreceptor genes are located within biodegradation gene clusters and are coordinately regulated with these genes (Pandey and Jain 2002; Parales and Harwood 2002). In cases where
the chemoreceptor has not yet been identified, chemotaxis toward different pollutants is
induced by growing on the pollutant itself or one of its metabolites, which likely encodes the
necessary chemoreceptors. Thus, it has been speculated that chemotaxis may be an integral
feature of the biodegradation process (Pandey and Jain 2002; Parales and Harwood 2002).
Genetic improvement of microorganisms has been suggested as an option for environmental
restoration (Samanta et al. 2002). Identification, isolation and characterization of chemotactic
bacteria coupled with genetically improved degradation ability may have great potential in
optimization of in situ bioremediation.

2.1.8. Quantification of chemotaxis for bioremediation

Both experimental and modeling approaches for quantification of chemotaxis with respect to
bioremediation enhancement are described in the literature (Marx and Aitken 1999; Marx, R. B.
and Aitken, M. D. 2000; Marx, R. B.; and Aitken, M. D. 2000; Pedit et al. 2002; Law and Aitken
2003; Olson et al. 2004; Hilpert 2005; Law and Aitken 2005; Long and Hilpert 2007) and will be
presented in this section.

2.1.8.1. Quantification of chemotaxis in bulk liquids

Marx and Aitken (1999) evaluated napthlene degradation by P. putida G7 in a typical capillary
assay experiment. Experimental data were fitted to a model in order to predict the chemotactic
sensitivity coefficient. The value obtained was found to be approximately three times lower
than previously reported values from a relatively simpler model (Barton and Ford 1995) that did
not account for the bacterial transport in the chamber. Another model that also incorporated
substrate consumption was able to predict chemotactic band formation, and replicated capillary
assay data well (Marx, R. B. and Aitken, M. D. 2000). Hilpert (Hilpert 2005) presents a numerical
modeling approach based on Lattice-Boltzmann methods for modeling bacterial chemotaxis and
the fate and transport of a chemoattractant in bulk liquid. Chemotactic traveling bacterial bands
in a uniformly distributed substrate region were simulated as a result of self-generated concentration gradients due to substrate consumption. Based on simulation results, they suggest that only a fraction of a bacterial slug injected into a chemoattractant domain forms a traveling band as the slug length exceeds a critical value. These findings are consistent with the capillary assay results from Adler [16].

2.1.8.2. Quantification of chemotaxis in saturated porous media

Pedit et al. (Pedit et al. 2002) measured the chemotactic response of *P. putida* G7 toward naphthalene in saturated porous media. To simulate saturated porous media, a conventional capillary assay method was modified by packing glass beads in the capillary tube and surrounding reservoir. A model was developed to estimate transport parameters including naphthalene diffusion, random motility, and chemotactic sensitivity. Simulations indicate that an order of magnitude higher cell concentration of the non-chemotactic strain would be required to achieve the same amount of naphthalene degradation as from a chemotactic strain. Chemotaxis in porous media systems can be approximated by free-liquid systems by accounting for soil parameters including tortuosity and porosity (Barton and Ford 1995). Recently, an analytical solution for bacterial chemotaxis in homogeneous porous media was presented by Long and Hilpert (Long and Hilpert 2007). They derived analytical solutions for chemotactic band velocities under different substrate input conditions. This approach could be important in comparing the chemotactic band velocity with groundwater velocity in order to assess the impact of chemotaxis on an overall remediation scheme.

Olson et al. (Olson et al. 2004) have used immunomagnetic labeling and magnetic resonance imaging (MRI) for non-invasive measurement of bacterial distributions in a packed column. Simulation of experimental data required addition of a non-zero chemotactic sensitivity term to account for the chemotactic response of *P. putida* F1 toward
trichloroethylene (TCE). In addition Olson et al. (Olson et al. 2006) mathematically demonstrated that bacteria traveling in a high permeability region with advective flow can successfully migrate toward and accumulate around contaminant diffusing from low permeability regions. The effect of pore size on transport parameters is also reported; a 50 % reduction in both motility and chemotaxis is reported for a similar reduction in pore size. These studies demonstrate that chemotaxis can potentially be exploited to enhance in situ bioremediation in the subsurface, particularly in heterogeneous and low permeability regions where low solubility contaminants such as NAPLs, remain trapped.

2.1.9. Impact of chemotaxis on contaminant degradation

A quantitative evaluation of enhanced chemoeffector degradation due to chemotaxis is presented by Law and Aitken (Law and Aitken 2003). Naphthalene desorption and degradation from a model NAPL was faster for a chemotactic wild-type P. putida G7 strain compared with non-chemotactic strains, which is attributed to the steep concentration gradient created by chemotactic bacteria near the NAPL surface. Thus, chemotaxis can be useful in increasing the rate of mass transfer and biodegradation of NAPL-associated hydrophobic pollutants.

The role of chemotaxis in naphthalene degradation by P. putida G7 in a heterogeneous aqueous system was evaluated experimentally by Marx and Aitken (Marx, R. B.; and Aitken, M. D. 2000). They demonstrated that mass transfer was a rate limiting step in naphthalene biodegradation by non-chemotactic strains. In contrast, the removal rate clearly exceeded the mass transfer rate in the case of the chemotactic wild-type strain of P. putida G7 and was approximately five times faster than the non-chemotactic strains. These results clearly indicate the possibility of enhancing bioremediation in aqueous systems by chemotaxis.
2.1.10. Bacterial motility assisted enhanced mixing

A large number of soil inhabiting bacteria such as *Escherichia coli* and *Pseudomonas putida* are able to independently swim in aqueous media by virtue of the rotation of a helical flagella bundle attached to their surfaces. Typical bacterial swimming consists of relatively straight runs of swimming, interrupted by random tumbling events that change the swimming direction (Berg and Brown 1972). This alternating series of runs and tumbles is governed by the direction of flagellar rotation that is controlled by regulatory motors at their base (Berg and Brown 1972; Berg 2004). The counterclockwise rotation of the motor pushes the bacteria forward during a run, while a change in direction of one or more motors breaks the flagella bundle resulting in the tumbling movement (Berg 2004). Thus bacteria provide a natural mechanism for mixing, and contaminant mixing can be achieved by putting bacteria in the appropriate environment (Berg 2003; Berg 2004; Kim and Breuer 2007a).

2.1.11. Effect of contaminant toxicity on viability of contaminant-degrading bacteria

It is well known that many ground-water contaminants that can be degraded by soil-inhabiting bacteria at low concentrations via metabolism or co-metabolism, are toxic to them above certain threshold concentrations (Collins and Daugulis 1999; Abuhamed et al. 2004; Kim et al. 2005). Toxicity of various cyclic hydrocarbons, such as those existing as non-aqueous phase liquids (NAPLs), is a critical factor that can limit or prevent their biodegradation (Sikkema et al. 1995). Prolonged exposure to higher concentrations of these contaminants may cause significant stress to bacterial cells, that may result in temporary or permanent loss of their ability to degrade specific contaminants (Kim and Jaffe 2007) or even in complete loss of cell viability. Such toxicity effects may be prominent in NAPL-contaminated aquifers where high contaminant concentrations may occur in the vicinity of the dissolving NAPLs. Contaminant toxicity to contaminant-degrading bacteria can adversely affect bio-enhancement by reducing
the proximity of bacteria to the contaminants via bacterial mortality. Bacterial viability is an important factor for contaminant removal as only viable bacteria are able to degrade contaminants and cells rendered non-viable may create adverse effects such as bio-clogging. Toxicity of contaminants to bacteria is related to their hydrophobicity and partitioning into membranes, which is closely correlated to the octanol-water partitioning coefficient, $K_{ow}$ of these compounds (Sikkema et al. 1994). Many organic compounds such as BTEX, toluene, TCE etc. that are major components of light and dense NAPLs, are hydrophobic ($1 < K_{ow} < 5$) and are extremely toxic to bacteria (Sikkema et al. 1994; Seagren et al. 2002).

2.2. Microfluidics

‘Microfluidics’ refers to the manipulation of fluids in networks of channels ranging from 5-500 µm that can handle fluid volumes as small as microliters ($10^{-6}$ l) to femtoliters ($10^{-15}$ l) (Weibel and Whitesides 2006; Whitesides 2006). A few sets of techniques divided into two major categories, photolithography and softlithography, are commonly used methods for fabricating microfluidic devices (MFDs). A general procedure consists of embossing the desired structure patterns by chemical deposition or etching on a silicon or glass surface. These patterns are subsequently transferred on the surface of a thin slab of polymer, usually poly(dimethylsiloxane) (PDMS), in academic laboratories (Duffy et al. 1998). Some other techniques such as dry- and wet- bulk micromachining, physical- and chemical- vapor deposition are also used in various other applications such as modification of the surface characteristics of the MFDs. Microfluidic devices (MFDs) are widespread in many scientific and industrial fluid manipulation applications including the areas of biology, analytical biochemistry, and chemical-biology, and have been applied to introduce mixing, pumping, and sorting fluids (Weibel and Whitesides 2006). Microfluidics has also found its way into environmental engineering and has been extensively
used in groundwater aquifer and marine research. The term ‘micromodels’ is interchangeably used for ‘microfluidic devices’ in the groundwater research literature.

2.2.1. Microfluidic device fabrication techniques

2.2.1.1. Photolithography

Photo-litho-graphy is derived from Latin, meaning light-stone-writing. Photolithography is an optical pattern transferring technique in which micro-patterns are transferred from a photomask to a UV-sensitive polymer layer (photoresist) coated on a substrate. It is a high resolution transferring technique and can be classified as E-beam lithography or X-ray lithography depending on the UV-light used.

2.2.1.2. Softlithography

The Whitesides group at Harvard University developed a non-photolithographic strategy based on self-assembly and replica molding for carrying out micro- and nanofabrication (Xia and Whitesides 1998). Softlithography technology is a term collectively used for the set of micro and nano-fabrication techniques including replica molding (REM), micro-contact printing (µCP), micro-molding in capillaries (MIMIC), micro-transfer molding (µTM), solvent assisted micro-molding (SAMIM), phase-shift photolithography, soft embossing (SE) etc (Xia and Whitesides 1998).

2.2.2. Microfluidics capabilities in environmental engineering

The advent of microfluidics is inspired by molecular analysis, molecular biology, and microelectronics (Whitesides 2006). Introduction of microfluidics in capillary format in analytical environmental chemistry techniques, such as gas-phase chromatography (GPC), high-pressure liquid chromatography (HPLC), polymerized chain reaction (PCR), have revolutionized the accuracy and precision of chemical analysis. The use of PDMS, which has several favorable properties, in microfluidics makes it possible to use them in a number of applications in
environmental engineering related to biological systems, involving cells and small microorganisms. PDMS is soft, flexible, biocompatible, electrically insulating, hydrophilic on oxidation, unreactive, transparent to visible and UV lights, permeable to gases and only partially permeable to water (Weibel and Whitesides 2006). The main disadvantage with PDMS is that it absorbs most of the organic solvents resulting in swelling and loss of its transparency, thereby changing its feature dimensions. Gas permeability may also impair its use with volatile organic compounds. Some of the possible applications of microfluidics in environmental engineering are described in the following sections.

2.2.2.1. Miniaturized prototypes

The science and technology of microfluidics provides easy and rapid prototyping in short time intervals, typically a day, with generation of multiple copies in a few hours with impeccable reproducibility. This ease of fabrication and adaptability enables researchers to easily simulate complex environmental systems in the lab. Microfluidic systems allow for spatial and temporal variation in reagents and nutrient addition to carry out desired reactions, replace media, and remove waste (Weibel and Whitesides 2006). As mentioned earlier, microfluidics uses very small devices that can handle tiny amounts of fluid volume and can therefore be used for manipulation of small amounts of samples allowing for isolation, visualization, and examination of very few or even single microorganisms (Lee et al. 2004; Wu et al. 2004; Balagaddé et al. 2005; Groisman et al. 2005; Cai et al. 2006). These characteristics are useful for in-situ observation of the microorganisms, and for physically isolating cells without changing their environment. In addition, the use of quantitatively small volumes of media and reagents in microfluidics is helpful in generating low waste, which is important in protecting the environment especially when working with hazardous materials (Lee et al. 2005). The miniaturized microfluidic prototypes of groundwater aquifers, which have sizes generally on the
order of few centimeters, have already begun to replace traditional column experiments and are being used extensively at both Darcy and pore scales (Sirivithayapakorn and Keller 2003a; Auset and Keller 2004; Keller and Sirivithayapakorn 2004; Auset et al. 2005; Werth et al. 2006; Lanning et al. 2008; Willingham et al. 2008; Long and Ford 2009).

2.2.2.2. Laminar flow conditions

As the physical dimensions of microfluidic channels decrease their surface-to-volume ratio increases, therefore microfluidic channels always have very high surface to volume ratios. As a result, flow in microfluidic channels is predominantly governed by viscous forces, resulting in low Reynolds number and laminar flow conditions. This characteristic of microfluidics is useful in studying natural and engineered environmental systems that automatically pose or require creation of such conditions. For example, microfluidic systems are useful in studying bacterial and contaminant transport in groundwater aquifers where laminar flow conditions naturally exist. Microfluidics is also beneficial in studies aimed at understanding cellular activity and behavior at the single cell scale.

2.2.2.3. Concentration gradients

Due to the laminar flow conditions in microfluidic devices, two or more fluid streams may flow side by side without any appreciable mixing of fluid particles, creating an experimental condition whereby diffusion is the only mixing mechanism between the fluids (Weibel and Whitesides 2006). This property of microchannels enables the creation of steep concentration gradients of small molecules between streams flowing parallel to each other, which may be extended over several orders of magnitude (Weibel and Whitesides 2006). These chemical gradients are usually perpendicular to the flow direction and have temporal and spatial stability. This is the single characteristic of microfluidic devices that has been exploited the most by researchers in environmental engineering and other areas for creating chemical gradients in an array of
complex designs. In environmental engineering, MFDs have been used for studying bacterial chemotaxis (Mao et al. 2003; Lanning et al. 2008; Long and Ford 2009), reactive-mixing transport (Willingham et al. 2008; Zhang, C. et al. 2010) and mineral precipitation in aquifers (Zhang, Changyong et al. 2010).

2.2.2.4. Environmental-Lab-on-a-Chip

Much of the work in microfluidics is focused on design and fabrication of the different components of a larger system. Current efforts are focused on integrating these components into a single device on a microchip plateform that can be automated for sample preparation, storing, and injection, chemical analysis (metering, sorting or mixing, detection), data acquisition, and waste rejection and disposal. Such automated devices are known as Lab-on-a-Chip systems or µTAS (micro total analysis systems) and are finding applications in the areas of biomedical sciences, clinical applications and homeland security and environmental monitoring of pollutants (Madou 2002). The primary products of microfluidics systems are expected to be used as sensors for detection of air and water pollutants via instruments known as microspectrometers, micro gas chromatography systems, micro ion mobility spectrometers, and infrared detectors (Madou 2002). The ability of this technology to produce repeated patterns of structures in a small space may be useful in prototyping complex environmental systems that require studying multiple processes at the same time.

2.2.3. Microfluidics applications in groundwater aquifer studies

One of the most prominent features of microfluidic devices - their ability to offer laminar flow conditions – naturally mimics groundwater flow in aquifers and has therefore been extensively exploited by groundwater researchers. Traditionally, soil column experiments were used for studying transport through porous media and the inherent ‘black-box’ approach was used for overall transport predictions. Such predictions have sometimes proved to be inaccurate due
their inability to account for micro scale processes that may affect the overall transport. The ability of microfluidic devices to facilitate direct observation at the micro scale has enabled a better understanding of the pore scale processes that govern biotic (i.e. viruses, bacteria, spores, and other microorganisms) and abiotic colloidal transport in porous media, thereby providing a better substitute for column experiments. Microfluidic devices used to simulate subsurface porous media range from PDMS molds to patterns etched on glass and silicon substrates and transparent flow cells. These devices have been used to simulate subsurface porous media for studying colloidal- (Wan and Wilson 1994; Auset and Keller 2004; Auset et al. 2005; Smith et al. 2008) and bacterial- (Lanning and Ford 2002; Lanning et al. 2008; Long and Ford 2009) transport, reactive-mixing transport (Willingham et al. 2008), biomass growth (Dupin and McCarty 2000; Stewart and Fogler 2001; Nambi et al. 2003), and mineral precipitation (Zhang, Changyong et al. 2010). Details on each of these studies are described in the following individual subsections.

2.2.3.1. Colloidal transport

Keller and Auset (2006) have provided an extensive review of the colloidal transport visualization techniques in porous microfluidic devices under saturated and unsaturated conditions. Colloidal transport studies in the literature have focused on studying diffusion of particles particularly with respect to their sizes relative to the pore dimensions and flow lines (Sirivithayapakorn and Keller 2003a; Auset and Keller 2004; Baumann and Werth 2004), particle exclusion (Sirivithayapakorn and Keller 2003a, b), attachment and detachment processes (Chen et al. 2002; Baumann and Werth 2004), and sorption at air-water-interfaces (AWI) and in unsaturated porous media (Wan and Wilson 1994; Sirivithayapakorn and Keller 2003a, b; Auset et al. 2005). It has been observed that larger colloids remain in the middle stream lines in the pore throats while smaller colloids tend to follow streamlines closer to the pore walls, resulting
in higher residence times and higher diffusivity in the porous media (Sirivithayapakorn and Keller 2003a; Auset and Keller 2004). This phenomenon may result in an increase in the velocity of the larger colloids, which can sometimes travel 1.5-3.0 times faster than the average pore water velocity (Keller and Auset 2006). The term ‘apparent dispersivity’ has been proposed to represent dispersion of colloids in porous media as dispersivity of the porous medium was found to be a function of colloidal size (Keller and Auset 2006). Pore throat to colloidal diameter ratio (T/C) has been reported as critical factor for colloidal exclusion processes and a threshold T/C value of 1.5 has been proposed for successful colloidal entrance into pore throats (Sirivithayapakorn and Keller 2003a). An empirical relationship proposed by Tufenkji and Elimelech (2004) indicates dependence of colloidal interception on the flow velocity; this inverse relationship was verified by Baumann and Werth (2004) in microfluidic experiments. The air-water-interface (AWI) is recognized as the single most important factor influencing colloidal transport in unsaturated porous media and microfluidic experiments have verified these locations as major regions of colloidal interception zones in unsaturated porous media (Wan and Wilson 1994; Sirivithayapakorn and Keller 2003a, b). Auset and Keller (2005) have reported that intermittent unsaturated flow helps in remobilization of bio-colloids from AWIs.

**2.2.3.2. Bacterial transport**

Researchers studying bacterial transport in groundwater have taken advantage of the property of MFDs that they do not provide any physical mixing between two fluid streams flowing in parallel whereby a sharp concentration gradient at the interface of the two streams is formed. Additionally, the size and transparency of microfluidic systems makes them an ideal tool for testing behavioral responses of microorganisms such as bacterial chemotaxis, which has been reported to influence groundwater bioremediation and biofilm formation in subsurface porous media. These characteristics of MFDs facilitate both individual cell tracking (such as cell
counting) and/or population based measurements (changes in light scattering intensity with bacterial concentration) that enable both qualitative and quantitative parameter determination. Ahmed et al. (Ahmed et al. 2010) have recently provided an extensive review of microfluidic gradient generators that have been used for studying bacterial chemotaxis and have emphasized the importance and potential of microfluidics for understanding the fundamental mechanism of bacterial chemotaxis as well as its application in realistic environment.

The earliest evidence of the use of microfluidics for chemotaxis measurement in relation to groundwater remediation is found in the device employed by Lanning and Ford (2002). They used micromodels etched on glass surfaces for studying bacterial dispersion in porous media under advective flow conditions. Chemotaxis was also measured under no-flow conditions. Mao et al. (2003)) pioneered a ‘T-sensor’ (named after its ‘T’ shape), an MFD in which a stream of bacterial suspension was introduced parallel to streams of a chemoeffector and a buffer suspension. The chemotaxis effect was assessed by observing bacterial migration toward the chemoeffector stream and was quantified in terms of the chemotactic sensitivity coefficient using eqs 2.11 and 2.12. Lanning and Ford (2008) used a modified ‘T-sensor’ in which three inlet streams were replaced by a two inlet stream device. The chemotactic effect was measured in terms of change in light scattering patterns due to shifts of bacterial concentration transverse to the flow direction and the effect was quantified in terms of a chemotactic sensitivity coefficient (eqs 2.11 and 2.12). Experiments were also preformed under stopped flow conditions and it was found that a mean flow velocity of 0.11 mm/s did not affect the chemotactic migration of the bacteria. Dual-bands of bacterial (E. coli HCB1) migration were also reported with 0.1 mM concentration of α-methylasparatate. An improvement in the MFD design proposed by Lanning et al. (2008) is presented by Long and Ford (2009) to more closely simulate the groundwater environment. A porous ‘T-sensor’ was employed in which a hexagonal arrangement of circular
cylinders of 200 µm diameter (representing soil grains) were added across the flow area of the device leaving pore throat spacings of ~46 µm between them. This design resulted in an equivalent porosity of 40%. Bacterial chemotaxis experiments were performed similar to those in Lanning et al. (2008) for flow velocities ranging from 5-20 m/day. Bacterial chemotaxis was measured in terms of change in bacterial count in the pore spaces between the simulated soil grains across the transverse cross-sections of the device. Similar to Lanning et al. (2008), numerical simulations were run to fit the experimental data to the bacterial chemotactic transport equations (Eq. 2.11 and 2.12) to quantify the chemotactic sensitivity coefficient. The authors concluded that the bacterial transport equations failed to provide accurate quantitative simulation of chemotaxis in the porous MFD and it was recognized that more work should be done to better understand bacterial transport in the porous media.

2.2.3.3. Reactive-mixing transport

Similar to the bacterial chemotaxis experiments, the ability of MFDs to form sharp impinging interfaces without any physical mixing between the two joining streams has been utilized by researchers for studying transverse-reactive-mixing transport in porous media. Willingham et al. (2008) used microfluidic experiments to verify that pore scale numerical simulations based on a lattice-Boltzmann finite volume model (LB-FVM) can accurately capture the physical and chemical processes that control transverse mixing-limited chemical reactions. MFDs with two inlet ports were used to introduce two reactive substrates in parallel streams, which underwent instantaneous reaction after coming in contact with each other in porous network of five different pore geometries. LB-FVM simulations were able to accurately capture the pore scale physical and chemical processes in MFDs. The interfacial contact area between two reactive species was reported as the most important factor controlling mixing and the extent of chemical reaction. The effect of gain size, grain orientation, and inter-particle porosity on the extent of
bimolecular reactions were also studied in the MFDs. Zhang et al. (2010) studied the effect of transverse-reactive-mixing on the growth of biomass in the porous MFDs, which will be discussed in the following section. Recently, MFDs have also been used for studying transverse mixing induced mineral precipitation and permeability reduction in relevance to groundwater remediation and geological carbon sequestration (Zhang, Changyong et al. 2010). Separate streams of CaCl₂ and Na₂CO₃ were introduced via two separate inlet ports to an MFD with a homogeneous pore structure; a precipitate of CaCO₃ was observed along the transverse mixing zone of the two streams at the center of the device. The pore spaces were occluded by the CaCO₃ precipitation along the transverse mixing zone and a significant reduction in the porosity and permeability of the device was observed.

2.2.3.4. Biofilm growth

Similar to mineral precipitation, biofilm formation in subsurface porous media is of concern from the prospective of porosity and permeability reduction and may adversely affecting the bioremediation of aquifers. Etched glass (Dupin and McCarty 2000; Nambi et al. 2003) and silicon (Kim and Fogler 2000; Stewart and Fogler 2001) micromodels (MFDs) have been used in earlier studies of biofilm growth in porous media. (Dupin and McCarty 1999, 2000) have used randomly distributed networks of balls and sticks for studying colonization of mixed cultures in porous media. Rerouting of flow due to biofilm growth was observed along with a decrease in conductivity. Decrease in permeability was observed due to strongly attached microorganisms to the surface of the device and each other. Continuous, over periodic, disinfection of biofilm was recommended. Kim and Fogler (1999) and Stewart and Fogler (2000) used a homogeneous triangular lattice of pore structures for biomass development in porous media. Biomass accumulation, evolution, and propagation in porous media were studied from the perspectives of bioremediation and oil recovery. Permeability reduction due to biomass growth in the porous
medium was observed and a critical shear stress existed for sloughing of biofilm. Exopolymer production led to biomass plug development which in turn caused a pressure drop across the MFD. A relatively modern and more refined version of the MFD was presented by Nambi et al (2003) for biomass growth in the transverse mixing zone of the two parallel flowing streams injected from two separate inlet ports in the device. It was observed that growth of biomass resulted in the change in water flow paths in the device along with increased velocity. A recent study in porous MFDs focused on the effects of pore heterogeneity and transverse mixing on biomass growth in porous media (Zhang, C. et al. 2010). Pore networks of homogeneous and heterogeneous grain arrangements in two-inlet MFDs with an additional port for disinfectant injection at the end of porous network were used. Overall, a relatively higher rate of biomass growth was observed in the MFD with a homogeneous pore network, as compared to a heterogeneous pore network, which the authors attributed to mass transfer limitation in the heterogeneous porous network.
CHAPTER 3: TRANSVERSE MIXING ENHANCEMENT DUE TO BACTERIAL RANDOM MOTILITY IN POROUS MICROFLUIDIC DEVICES*

3. Abstract

Bacterial swimming in groundwater may create flow disturbances in the surrounding microenvironment, thereby enhancing contaminant mixing. Porous micro-fluidic devices (MFDs) were fabricated in three different pore geometry designs: uniform grain size with large pore throats (MFD-I), non uniform grain size with restricted pore space (MFD-II), and uniform grain size with small pore throats (MFD-III). *E. Coli* HCB33 was used to assess the effect of bacterial random motility on transverse mixing of a tracer, fluorescent labeled Dextran, under three experimental conditions in which motile bacteria, non-motile bacteria, and plain buffer suspensions were flown through the MFDs at four different flow rates. Mixing was quantified in terms of the best-fit effective transverse dispersion coefficient \((D_{cy})_{eff}\). A mixing enhancement index (MEI) was defined as the ratio of the \((D_{cy})_{eff}\) of tracer in experiments with motile bacteria and without bacteria. Motile bacteria caused a maximum 5-6 fold increase in MEI in MFD-II, a nearly 4-fold increase in MFD-I and very little observed change in MFD-III. The apparent transverse dispersivity \((\alpha_{app})\) of MFD-II and MFD-I increased by 3 and 2.3 times, respectively, with no change in MFD-III. These observations indicate that both, pore throat size and pore arrangement are critical factors for contaminant mixing in porous media.

*Most of the material contained in this chapter is accepted for the publication in *Environ. Sci. Technol.*, DOI: 10.1021/es201706w
3.1. Introduction

The presence of soil grains introduces tortuosity in groundwater flow paths, resulting in velocity gradients which may enhance transverse contaminant mixing (Ajdari et al. 2005). For situations in which contaminant mixing is controlled by molecular diffusion, the transverse mixing zone width may be as small as on the order of millimeters or centimeters (Acharya et al. 2007). Transverse contaminant mixing is critical at the fringes of contaminant plumes, which form sharp concentration gradients as a result of dilution with surrounding groundwater (Cirpka et al. 1999; Huang et al. 2003; Rahman et al. 2005; Cirpka et al. 2006; Werth et al. 2006; Acharya et al. 2007; Knutson et al. 2007; Willingham et al. 2008). Under steady-state flow conditions, transverse dispersion may enhance biodegradation of contaminant plumes by i) replenishing depleted electron acceptors in the plume zone (Rahman et al. 2005), ii) diluting contaminant concentrations, thereby creating a potentially more favorable degradation environment (Abuhamed et al. 2004; Kim et al. 2005; Ray and Peters 2008; Singh and Olson 2010), and iii) promoting activities such as chemotaxis through formation of chemical gradients at plume fringes.

Many soil-inhabiting bacteria such as *Escherichia Coli* (*E. coli*) and *Pseudomonas putida* (*P. putida*), are capable of swimming in aqueous solutions via flagella attached to their surfaces. A typical swimming mechanism of flagellated bacteria in aqueous media is characterized as a series of alternating run and tumbling motions, which are propelled by 6-8 rotating helical filaments (flagella) protruding from the cell surfaces (Ford and Harvey 2007). The rotation of flagella is governed by a flagellar motor at their base. Counterclockwise rotation of motors and hence flagella tends to form a coordinated bundle behind the cell body and pushes the cell to move forward smoothly. When one or more motor changes its rotational direction, the flagella bundle unravels and the cell tumbles chaotically and reorients itself to move in another
direction (Turner et al. 2000; Ford and Harvey 2007). Flagellar motors have a unique feature in that they alternate direction between clockwise to counterclockwise in a random manner and therefore overall bacterial motion results in a 3D random walk in the surrounding fluid (Kim and Jaffé 2007) that resembles molecular diffusion of gases (Ford and Harvey 2007). This chaotic movement of bacteria creates a natural mechanism for mixing in their micro-surroundings and may be exploited to engineer an enhancement of overall mixing (Kim and Breuer 2007a) which has been demonstrated in several studies (Wu and Libchaber 2000; Darnton et al. 2004; Kim and Breuer 2004, 2007a, b; Sokolov et al. 2009; Al-Fandi et al. 2010).

In the past decade, the novel idea of chemical mixing enhancement due to bacterial flagellar rotation has been explored in different applications. Mixing enhancement due to freely swimming *E. coli* in aqueous suspensions is reported as a result of bacterial random motility (Kim and Breuer 2004) and chemotaxis (Kim and Breuer 2007a), where effective diffusion coefficients are observed to increase linearly with increases in bacterial and attractant concentrations, respectively. Kim and Breuer (2007) reported a greater than 30-fold increase in the effective diffusion coefficient of a passive tracer as a result of the random rotation of the flagella of a *Serratia marcescens* carpet attached to the surface of a microfluidic system. Al-Fandi et al. (2010) studied mixing due to engineered tethered *E. coli* cells and found that mixing enhancement was the result of cell body rotation around a single shortened flagellum attached to a surface. In other studies, diffusion enhancement of fluorescent tracer particles (Darnton et al. 2004) and micron-scale beads (Wu and Libchaber 2000) was observed due to bacterial carpets and freely swimming bacteria, respectively. Mixing enhancement as a result of bacterial locomotion in fluids in some of these studies (Wu and Libchaber 2000; Kim and Breuer 2004, 2007a) is attributed to superdiffusion, a nonconventional diffusion process that accounts for bacteria-bacteria interactions in aqueous media and results in chemical mixing that is faster
than would be predicted with a conventional Fickian diffusion model where the concentration gradient is considered as the only driving force.

The central focus of this paper is on mixing enhancement in porous media due to bacterial motility, which to the best of author’s knowledge has not yet been explored. This study compares the apparent transverse dispersivity in microfluidic devices with varying pore geometries in chemical transport experiments with motile bacteria, non-motile bacteria, and without bacteria. Polydimethylsiloxane (PDMS) microfluidic devices (MFDs) have been used in many scientific and industrial fluid manipulation applications, and may be used to measure mixing and dispersion at the sub-millimeter scale (Stone et al. 2004). Numerous groundwater studies (Auset and Keller 2004; Auset et al. 2005; Werth et al. 2006; Lanning et al. 2008; Willingham et al. 2008; Long and Ford 2009) have successfully used these devices for quantification of pore scale parameters covering various aspects of groundwater remediation research such as colloidal (Wan and Wilson 1994; Auset and Keller 2004; Auset et al. 2005; Smith et al. 2008) and bacterial (Lanning and Ford 2002; Lanning et al. 2008; Long and Ford 2009) transport, contaminant mixing (Willingham et al. 2008), and biomass growth (Dupin and McCarty 2000; Stewart and Fogler 2001; Nambi et al. 2003) in porous media. In this study, we employ a novel two dimensional bi-layer porous microfluidic device (MFD) that simulates formation of a plume in an aquifer due to leakage of an underground storage tank for quantification of mixing enhancement due to bacterial motility in porous media. Mixing enhancement is quantified by comparing the effective dispersion coefficient of a model contaminant in experiments in the presence and absence of suspended motile bacteria. The effects of pore geometry and permeability of porous media on contaminant mixing are also analyzed by comparing the effective transverse diffusion coefficients and apparent transverse dispersivities in three microfluidic devices at four separate flow velocities.
3.2. Materials and Methods

3.2.1. Design and fabrication of microfluidic devices (MFDs)

The microfluidic device designed and fabricated for this study simulates an environmentally relevant scenario of groundwater contamination due to leakage from an underground storage tank (UST). A schematic of the device is shown in Figure 3.1, which shows the two PDMS layers, one on top of the other, bonded to a cover glass. The lower PDMS layer, with imprinted porous media, simulates a very thin layer (~0.022 mm) of a two-dimensional aquifer and forms a confined porous channel with the cover glass. The non-porous channel on the top, formed between two PDMS layers, simulates continuous leakage of contaminant from an UST and is connected to the lower porous channel at its downstream end through an orifice at the center of the porous channel. Further design details of the device are shown in Figure 3.1.

The microfluidic devices (MFDs) were fabricated using standard photolithography and soft lithography techniques (Duffy et al. 1998; Whitesides and Stroock 2001) with appropriate modifications. Masks were designed using AutoCAD2008 to simulate a homogeneous two-dimensional porous medium (see Figure 3.2 and pore geometries section for details). A high-resolution printer (20000 dpi) printed this design on a transparency (CAD/Art Services, Inc., Bandon, OR) that served as a mask for the subsequent photolithography process. A thin layer (~22 µm) of negative photoresist SU-8 (microchem) was coated (Specialty Coating System Inc., G3P-8 Spin Coat) on a cleaned 3” silicon wafer (Wafer World, Inc.). The coated layer was baked at 65 °C for 1 min, the temperature was gradually increased to 95 °C to provide uniform heating, and then the coated layer was further baked at this temperature for 5 min. In order to transfer the patterns onto the SU-8 coated silicon wafer, it was exposed to an appropriate dose
Figure 3.1: Schematic representing the bi-layer micro-fluidic device (MFD) used for testing contaminant mixing enhancement due to bacterial motility in porous media. An isometric-view (top) showing the porous channel (between the glass and first PDMS layers) simulating a very thin layer of a two dimensional aquifer slice connected to an overlying channel (between two PDMS layers) simulating leakage from an underground storage tank. A vertical cross-sectional view through section AA’ (bottom) shows the closed channel opening though which different injectates flow through the device. Cross-sections representing sampling locations at 4.9, 9.8, 19.6, 29.2, and 36.75 mm downstream from the tracer inlet point into the porous channel are also shown (top). A representative zoomed-in view at a cross-section is also shown on the right. Specific details of different devices are presented in Table 3.1.
(≈1.6 mJ/mm²) of UV light (Karl Suss MJB-3) with mask at the top. UV exposed areas of the coated SU8 layer crosslink to the silicon wafer resulting in a permanent bond, and the remaining unexposed SU-8 layer was removed by developing SU-8 developer (michrochem). The sample was briefly sonicated (FS30 Fisher Scientific) during the developing process to improve unexposed SU-8 removal, thereby ensuring better finishing of the microstructures. This process resulted in the final mold that was subsequently used in the soft lithography process for printing the patterns onto PDMS.

The mold was coated with T2492-KG (Uct Specialties, Llc) using vapor deposition in a vacuum desiccator (Ted Pella inc.) for 30 min. A 9:1 mixture of 184-Sylgaurd elastomer base and curing agent (Dow Corning) was mixed thoroughly and degassed under 1 PSI vacuum to remove the trapped air bubbles. Degassed mixture was poured onto the T2492-KG coated mold and heat cured at 70 °C (Fisher Scientific® Isotemp Hotplate are Stirrer) for 1-hour to solidify the mixture. The solidified PDMS layer was peeled-off from the mold, and holes were punched in the wells to create the inlets (Figure 3.1). In order to form closed confined channels for bacterial and contaminant injection, the part of the PDMS layer containing the porous channel was bonded with a cleaned glass microscopic slide via plasma treatment (Harrick Plasma cleaner) for 45 sec at high treatment mode. Similarly, the PDMS layer containing the small non porous channel was bonded at the top of the porous channel with appropriate alignment as shown in Figure 3.1. For each set of experiments a new device was used to prevent biomass clogging of the device and experiments were performed within a few hours of device fabrication to ensure hydrophilicity of the surfaces and maintain consistency across the experiments.

3.2.2. Pore geometries

Three homogeneous pore geometries representing three pore structures used in this study are shown in Figure 3.2. The first porous media design consists of a regular pattern of uniform dark
circles of diameter 0.300 mm (representing soil grains) spaced 0.350 mm apart and therefore leaving a pore throat spacing of 0.050 mm between them (Figure 3.2A). A second design consists of a similar pattern of dark circles, along with one smaller circle of 0.100 mm right in the middle of the pore space resulting from the four larger circles (Figure 3.2B). These two designs in Figures 2A and 2B result in porosities of 42.3% and 35.9%, respectively. To study the effect of pore geometry and porosity on contaminant mixing enhancement a third pore geometry design was selected with a pore grain design similar to the first design and equivalent porosity to the second pore design. This porous media design resulted in pore throat spacing of 0.032 mm (Figure 3.2C). For future discussion, the microfluidic devices fabricated using the first, second, and third designs will be referred to as MFD-I, MFD-II, and MFD-III, respectively. Rough surfaces of the simulated soil grains are evident from Figure 3.2D and offer a better representation of soil grains. The Kozeny-Carman equation (McCabe et al. 2005) was employed to estimate the permeability (k) of the devices, as reported in Table 3.1:

\[
\frac{2}{2} \left( \frac{3}{150} \right)^2 \]

where, \( \phi_s \) is sphericity of the grains (\( \phi_s = 1 \) for this study), \( D_p \) is grain diameter, and \( \varepsilon \) is porosity.

3.2.3. Cell culture preparation and chemicals

*Escherichia coli* HCB33 (wild type strain) cells were obtained from Dr. Min Jun Kim, Drexel University. Cells from a -80 °C frozen glycerol stock were grown on Luria broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter of DI water) in a shaking incubator @ 200 rpm (VWR Incubating Orbital Shaker) at 33 °C for 4 h. Cells were harvested in their exponential growth phase at an optical density at 590 nm (OD590) of ~ 0.8-1.0 (Spectronic® 20 GENESYS™ Spectrophotometer). Cells were washed with 10 % random motility buffer (RMB) (11.2 g
K$_2$HPO$_4$, 4.8 g KH$_2$PO$_4$, and 0.029 g EDTA per liter of DI water; pH ~ 7.0) by centrifugation and resuspended in buffer to a final concentration of OD$_{590} = 0.60$ (~1.5x10$^9$ cells/ml). For experiments in which non-motile bacteria were required, FCCP (carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone), was added (0.001% w/v) to 10% RMB, to deenergize the flagellum rotary motor and render the cells non-motile (Kim and Breuer 2004).

A relatively high molecular weight diffusive tracer, fluorescein isothiocyanate labeled Dextran (FITC-Dextran) (Molecular Probe, Invitrogen), average molecular weight 70000, was used in this study for quantification of mixing enhancement. Kim et al (Kim and Breuer 2007a) recommend the use of higher molecular weight tracers for determination of effective diffusion coefficients to avoid a dramatic concentration deflection common with smaller tracer molecules.

3.2.4. Experimental set up

To determine the effect of bacterial random motility on transverse mixing in porous media, three types of contaminant transport experiments were performed by injecting three different injectates into the porous bottom channel (through port A, Figure 3.1) of the MFDs: (1) motile bacterial suspension in 10% RMB, (2) non-motile bacterial suspension in 10% RMB, and (3) 10% RMB only. A low concentration (0.02 % (w/v)) of FITC-Dextran solution in 10% RMB was simultaneously injected through the top channel (port B, Figure 3.1) in all three type of experiments. A CCD camera (AxioCam, Carl Zeiss) mounted on a stereoscope (Zeiss Stereo Discovery V8 equipped with GFP-470/525 filter, 3.2x magnification) was used to capture images at 80 ms exposure time at transverse cross-sections 4.9, 9.8, 19.6, 29.4, and 36.75 mm downstream from the point of FITC-Dextran injection into porous channel (Figure 3.1) in all three experiments.
Figure 3.2: Images showing zoomed-in pore geometry details of the three pore designs used in this study- a regular pattern of uniform grain size (MFD-I), a regular pattern of two different grain sizes (MFD-II), and a regular pattern of uniform grain size with equivalent porosity of MFD-II (MFD-III). Bottom right side picture shows a macroscopic image taken at 63X magnification inside a pore body of MFD-II. The larger grain diameter in all devices (dark black circles in A, B, and C) is 0.300 mm and smaller grain diameter in MFD-II (smaller black dots in B) is 0.100 mm. Pore throat spacing in MFD-I and MFD-II is 0.050 mm and in MFD-III is 0.032 mm.

Table 3.1: Summary of apparent dispersivity ($\alpha_{app}$) values for three devices under different experimental conditions.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Transverse dispersivity coefficients ($\alpha_{app}$), mm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uniform grain-size media (MFD-I)</td>
<td>Non-uniform grain-size media (MFD-II)</td>
</tr>
<tr>
<td></td>
<td>$\varepsilon = 42.3%$</td>
<td>$\varepsilon = 35.9%$</td>
</tr>
<tr>
<td></td>
<td>$k = 1.36 \times 10^{-4} \text{mm}^2$</td>
<td>$k = 0.44 \times 10^{-4} \text{mm}^2$</td>
</tr>
<tr>
<td></td>
<td>$D_{eff} = 4.86 \times 10^{-6} \text{mm}^2/\text{s}$</td>
<td>$D_{eff} = 4.13 \times 10^{-6} \text{mm}^2/\text{s}$</td>
</tr>
<tr>
<td>Motile Bacteria</td>
<td>0.0048±0.0006</td>
<td>0.0038±0.0008</td>
</tr>
<tr>
<td>No Bacteria</td>
<td>0.0021±0.0004</td>
<td>0.0013±0.0003</td>
</tr>
<tr>
<td>Non-motile Bacteria</td>
<td>0.0028±0.0003</td>
<td></td>
</tr>
</tbody>
</table>

*Value is calculated based on the geometric mean grain size of the pore design shown in Figure 3.2.*
3.1.1. Microfluidic device (MFD) operation

A multiple loading syringe pump (kd Scientific, Model 200 Series) was used for injecting two injectates, depending on the type of experiment, into the two ports of the MFD. The top channel was connected to a 1-mL syringe (BD, internal diameter 4.77mm) and the bottom channel was connected to 10-mL syringe (BD, internal diameter 14.50 mm), resulting in a volumetric flow rate ratio of 9.2 : 1 in the bottom : top channels. Polyethylene tubing (INTRAMEDIC® Polyethylene Tubing, Clay Adams®) was used to attach the punched inlets of the channels to their respective syringes via Syringe needles (BD 21G1). The high volumetric flow rate ratio was chosen to mimic groundwater and contaminant volumetric flow ratios in actual groundwater aquifers. Experiments were performed at Darcy velocities (calculated by dividing volumetric flow rates by the total cross-sectional area of the flow channel) of 0.11, 0.22, 0.44, and 0.88 mm/s, a range chosen from similar groundwater studies in micromodels (Willingham et al. 2008; Long and Ford 2009). Equilibrium flow conditions were ensured by allowing at least two pore volumes of fluid to flow through the device before taking measurements.

3.1.2. Mathematical modeling

3.1.2.1. Transverse dispersion coefficient: The two-dimensional advection-dispersion equation that governs transport of a tracer in porous media may be given by the following mathematical expression:

\[
R_c \frac{\partial C}{\partial t} = -v_f \frac{\partial (C)}{\partial x} + \left( (D_{cx})_{\text{eff}} \frac{\partial^2 C}{\partial x^2} + (D_{cy})_{\text{eff}} \frac{\partial^2 C}{\partial y^2} \right)
\]

where, \( R_c \) is retardation factor, \( C \) is tracer concentration, \( t \) is time, \( x \) and \( y \) are longitudinal and transverse directions to flow direction, \( v_f \) is average linear flow velocity, \( (D_{cx})_{\text{eff}} \) and \( (D_{cy})_{\text{eff}} \) are effective longitudinal and transverse dispersion coefficients, respectively. For a conservative tracer \( R_c=1 \). At steady-state flow conditions the longitudinal concentration gradient is small,
therefore diffusive mixing in the longitudinal direction is also small in comparison to the
advective transport term and can be neglected (Long and Ford 2009). The tracer concentration
was linearly related to fluorescent intensity, I, of the FITC-Dextran and could therefore be
substituted in Eq 3.2. Under these assumptions, Eq 3.2 is reduced to:

\[ v_f \frac{\partial (I)}{\partial x} = (D_{cy})_{eff} \frac{\partial^2 I}{\partial y^2} \]  \hspace{1cm} 3.3

The analytical solution to Eq 3.3 was derived from 1D solute diffusion from a confined region of
width h at the tracer inlet into the porous channel and is given by (Crank 1975):

\[ \frac{I}{I_0} = \frac{1}{2} \left\{ \text{erf} \left( \frac{h}{2} - y \right) \frac{h}{2 - y} + \text{erf} \left( \frac{h}{2} + y \right) \frac{h}{2 + y} \right\} \]  \hspace{1cm} 3.4

where, \( I_0 \) is the peak fluorescent intensity. Experimentally obtained images were analyzed
for the fluorescent intensity distribution at cross-sections 4.9, 9.8, 19.6, 29.4 and 36.75
mm downstream from the FITC-Dextran inlet point into the porous bottom channel
using an image processing program (Image J) developed by the National Institute of
Health. For the sake of consistency, the intensity profiles obtained at each cross-section
were normalized with respect to the corresponding peak intensity value, as opposed to
the peak intensity at the inlet. It was not possible to measure the maximum intensity (I_0)
near the tracer inlet (Figure 1) due to light interference from the tracer inlet channel.

To obtain the effective transverse dispersion coefficients, normalized steady state
transverse intensity profiles, were fitted to the analytical solution given by Eq 3.4. Due to
symmetry of the device, the intensity profiles can be cut in half by a vertical plane at \( y=0 \), and
therefore only half of the porous channel region \( (y \geq 0) \) was chosen for modeling the fluorescent
intensity profiles. The tracer plume width, \( h \), was measured immediately downstream (\( x = 1.05 \) mm) from the point of injection of the tracer into the porous bottom channel and was assumed to be constant at that cross-section (\( x = 1.05 \) mm) due to continuous injection of FITC-Dextran (\( l[x\leq1.05 \text{ mm}, -h/2sysh/2, t\geq t_{\text{equlb}}]= l_0 \)), where \( t_{\text{equlb}} \) is the time required to attain equilibrium flow conditions. The total width of the device (6.3 mm) was approximately three times the maximum plume width observed during the experiments, and therefore channel boundaries were approximated as infinite distance, zero concentration boundary conditions.

3.1.2.2. Apparent transverse dispersivity: The effective transverse dispersion coefficient in porous media is given by:

\[
(D_{cy})_{\text{eff}} = (D_0)_{\text{eff}} + \alpha_{\text{app}} v_f
\]

where, \( (D_0)_{\text{eff}} \) is effective diffusion coefficient and \( \alpha_{\text{app}} \) is apparent transverse dispersivity of the porous medium. Both, \( (D_0)_{\text{eff}} \) and \( \alpha_{\text{app}} \), are lumped parameters that account for the effect of the presence of bacteria and porous media in the system. The effective diffusion coefficient of the tracer can be given by (Sherwood et al. 2003; Olson et al. 2005)

\[
(D_0)_{\text{eff}} = \frac{\varepsilon}{\tau} D_0
\]

where, \( \varepsilon \) and \( \tau \) are porosity and tortuosity of the porous medium and \( D_0 \) is the molecular diffusion coefficient of the tracer. The porosity (\( \varepsilon \)) values for different porous media are reported in Table 3.1. The tortuosity term incorporates the effects of the available pore space and the transport mechanism of the tracers (Olson et al. 2005) and an assumed value of 2 will be used in this study based on values used in similar groundwater studies (Acharya et al. 2007; Long and Ford 2009). The molecular diffusion coefficient of the tracer FITC-Dextran is \( D_0=2.3 \times 10^{-5} \text{ mm}^2/\text{s} \) (Periasamy and Verkman 1998).
3.2. Results

3.2.1. Comparison of FITC-Dextran intensity profiles

A fluorescent plume along the length of the device was apparent as FITC-Dextran entered and flowed through the porous bottom channel. At steady state flow conditions, images of the plume were recorded at the pre-defined cross-sections (Figure 3.1). Figure 3.3 shows representative images at cross-sections 9.8 mm and 29.4 mm downstream from the FITC-Dextran inlet in MFD-I under different experimental conditions. Transverse dispersion of the plume is evident from the increase in the width of fluorescent intensity profiles from 9.8 mm (row 1, Figure 3.3) to 29.4 mm (row 2, Figure 3.3) in all three experimental conditions (Columns 1 to 3). Comparison of concentration profiles under different experimental conditions (across different columns) shows wider profile widths in experiments with motile bacteria (column 1, Figure 3.3) than in experiments with no bacteria (column 2, Figure 3.3) at respective cross-sections. To further verify that the effect was due to bacterial motility, control experiments were performed in which the motile bacteria were replaced by similar concentrations of non-motile bacteria in a third set of experiments. The widths of the fluorescent intensity profiles in experiments with non-motile bacteria (column 3, Figure 3.3) are not significantly different from the corresponding profile widths in experiments with no bacteria (column 2, Figure 3.3). These observations indicate that the wider profile widths in experiments with motile bacteria are the result of the random motility of *E. Coli* HBC33 in porous media.

3.2.2. Effective transverse dispersion coefficients

Fluorescent intensity distributions were analyzed at cross-sections 4.9, 9.8, 19.6, 29.4 and 36.75 mm downstream from the tracer inlet point in the porous bottom channel of the MFDs. The experimentally obtained representative normalized intensity profiles for MFD-I 29.4 mm downstream from the inlet under various experimental conditions are shown in Figure
**Figure 3.3:** Typical fluorescein isothiocyanate labeled dextran (FITC-Dextran) dispersion images at cross-sections 9.6 mm and 29.4 mm downstream from the tracer inlet point in the porous channel at Darcy velocity 0.22 mm/s. The overlaid profiles in each image indicate the fluorescent intensity variation across the channel between two horizontal rows of simulated soil grains.
3.4. The best-fit dispersion coefficient values at each cross-section of the device were obtained by fitting the model (Eq 3.4) to the corresponding experimentally obtained normalized intensity profiles using least squares regression analysis. Average transverse dispersion coefficients (ATDC) for each cross-section were calculated by averaging the best fit values of triplicate experiments. ATDC values obtained at Darcy velocities of 0.11 mm/s and 0.22 mm/s in MFD-I at different cross-sections are shown in Figure 3.5 during mixing experiments with motile bacteria.

The values of ATDC at cross-section 4.9 mm from the inlet are approximately an order of magnitude higher than the dispersion coefficient values at the rest of the downstream cross-sections. The ATDC values further decrease at cross-section 9.8 mm and eventually level off to approximately a constant value for remaining three cross-sections. Larger values of ATDC at cross-sections 4.9 mm and 9.8 mm are accompanied by relatively large error bars at these cross-sections which indicate larger variability across the experiments. Due to the large disparity in the ATDC values at the two upstream cross-sections, these values were not included in the effective transverse dispersion coefficient calculations for the device.

\((D_{cy})_{eff}\) for the device under different experimental conditions was calculated by averaging the ATDC values at cross-sections 19.6, 29.4, and 36.75 mm downstream from the inlet, where these values were nearly constant (Figure 3.5). The effective transverse dispersion coefficient values were obtained as the global average of triplicate experiments. The model-predicted (Eq 3.4) profiles for the effective transverse dispersion coefficients for MFD-I at a cross-section 29.4 mm downstream from the inlet under various experimental conditions are shown in Figure 3.4 along with the corresponding experimentally obtained profiles. The best fit curves to the experimentally obtained effective transverse dispersion coefficient data for MFD-I are presented in Figure 5 under three experimental conditions.
Figure 3.4: Markers represent FITC-dextran normalized intensity profiles in MFD-I at a cross-section 29.2 mm downstream from its inlet in experiments with motile bacteria (left plot), immobilized bacteria (middle plot), and no bacteria (right plot). Each marker represents pixel intensity averaged over ten pixels and the curves represent corresponding modeled profiles for the respective effective transverse dispersion coefficients obtained as the global average of triplicate experiments.
Figure 3.5: Average transverse dispersion coefficient ($D_{cy}$) values at different cross-sections of the device, estimated based on the average of three best fit model values for three experimental profiles at each cross-section in mixing experiments with motile bacteria. The x-axis represents longitudinal distance of the cross-sections from the inlet of FITC-Dextran into the porous bottom channel. Error bars indicate standard error from triplicate experiments.
3.1.1. Apparent transverse dispersivities

Effective diffusion coefficients for three porous media geometries were calculated (Eq. 3.6) and the values obtained are reported in Table 3.1. Apparent transverse dispersivity values under each experimental conditions in each MFD were calculated using Eq. 3.5 given the effective diffusion coefficient values along with the best-fit effective transverse dispersion coefficients, \((D_{cy})_{eff}\), obtained in the previous section. Least square regression analysis was used to determine the best-fit values of the apparent dispersivity, \(\alpha_{app}\), for triplicate experiments.

Apparent dispersivity values for MFD-I under three experimental conditions are reported in Table 3.1. Compared with experiments run with no bacteria, it can be observed that the presence of motile bacteria results in a 2.5-fold increase in the apparent transverse dispersivity of the device. The presence of non-motile bacteria also resulted in a marginal (1.3 times) increase in the apparent transverse dispersivity of the device (Table 3.1), which may be attributed to two factors.

3.1.2. Effect of pore geometry on average- and effective- transverse dispersion coefficients

The ATDC values for MFD-II at Darcy velocities 0.11 mm/s and 0.22 mm/s at various cross-sections are shown in Figure 3.5. Similar to MFD-I, ATDC values for MFD-II at cross-sections closer to the inlet (at 4.9 and 9.8 mm) are significantly higher than the values for the remaining three downstream cross-sections, where ATDC values were nearly constant. Similar observations were made for MFD-III as well, and effective transverse dispersion coefficient values for MFD-II and MFD-III were calculated by averaging ATDC values at three downstream cross-sections, as in the case of MFD-I. Apparent transverse dispersivities and effective diffusion coefficient values were also calculated for MFD-II and MFD-III as described for MFD-I and the resulting values are presented in Table 3.1. A three-fold increase in the apparent transverse
dispersivity was observed in experiments with motile bacteria as opposed to no bacteria in MFD-II, while no such increase was observed in MFD-III.

3.2. Discussion

Hydrodynamic dispersion results in an increase in the variance of a tracer in porous media as it moves downstream from its injection point. This phenomenon is responsible for the spreading of fluorescent intensity profiles from cross-sections 9.8 mm (column 1, Figure 3.3) to 29.4 mm (column 2, Figure 3.3) downstream of the inlet in all three experimental conditions. Wider profiles widths were observed at each cross-section in the presence of motile *E. Coli.* HCB33 (row 1, Figure 3.3) as compared to experiments with no bacteria (row 2, Figure 3.3). However, the profile widths at each cross-section in the experiments with non-motile bacteria (row 3, Figure 3.3) were similar to those in experiments with no bacteria. Best fit effective dispersion coefficient values display a similar trend, with largest values for the experiments with motile bacteria and approximately similar values for experiments with non-motile bacteria and no bacteria (Figure 3.6). These two observations indicate that random motility of *E. Coli.* HCB33 does enhance mixing of FITC-Dextran in the porous MFDs. The marginal differences in the effective dispersion coefficient values in experiments with non-motile bacteria and no bacteria (Figure 3.6) may be attributed to the pore space occupied by the non-motile bacteria (bacterial concentration used in this study accounts for approximately ~ 0.36% of the total available pore space (calculated based on the values reported by Kim et al. (Kim and Breuer 2004) in similar studies)) or to the non-Fickian (or anomalous) diffusion of bacteria in porous media (Berkowitz et al. 2006; Bijeljic et al. 2011).

In general, the best fit models for the effective dispersion coefficients capture the shapes of experimentally obtained profiles well (Figure 3.4). However, the minor disparity between model predictions and experimentally obtained values at the lower tails of the curves (Figure 3.4), may
be attributed to non-Fickian behavior due to bacterial motility or to experimental limitations, including non-ideal inlet conditions and/or pump fluctuations. Another potential source of error between experimental data and modeled predictions may have been introduced when intensity profiles at downstream cross-sections were normalized with respect to their local maximum intensity rather than the peak intensity at the inlet. Although the peak intensity values at all cross-sections were analyzed and no significant change was observed among the various cross-sections, it is still possible that the normalization process was a source of error.

It is expected that an inertia will be associated with the FITC-Dextran particle cloud as it is released from the top channel into the porous bottom channel in MFDs (Figure 3.1). This inertia in the flow particles from the top channel may result in a perturbation to the overall flow in the porous bottom channel near the FITC-Dextran inlet point. This perturbation in flow was evident in channelized fluorescent flow patterns observed in images near the inlet point and as expected, the effect was more pronounced at higher Darcy flow velocities. Fisher et. al (Fischer 1979) have reported that for a short time after the release of the dispersant into the flowing stream, known as ‘Lagrangian time’, the variance of the particle ensemble averaged concentration distribution grows as $t^2$. In the case of a Gaussian distribution (which is the base of the model used in this study (Eq 3.4)), variance of the concentration distribution grows linearly with time. Given the abnormally high ATDS values at the two upstream cross-sections (Figure 3.5), it is likely that the proximity of the two upstream cross-sections to the inlet may result in the lack of sufficient ‘Lagrangian time’, for the particle cloud to lose its initial velocities, thereby resulting in the high ATDC values at these cross-sections. Acharya et al. (Acharya et al. 2007) have also reported similar discrepancies in the dispersion coefficient values near the inlet in 2-D pore scale simulation studies.
Figure 3.6: Variation of effective transverse dispersion coefficient, $(D_{cy})_{eff}$, with Darcy velocity in MFD-I under three different experimental conditions. Error bars represent standard error values of nine best fit profiles at three cross-sections of the device in triplicate experiments. The line represents results of the dispersion model (Eqn 3.6) with the best fit model parameter values given in Table 3.1.
The effect of pore geometry on ATDC variation in micro-fluidic devices is shown in Figure 3.5. It can be observed that the disparity in the ATDC values at two upstream cross-sections for MFD-II are relatively smaller than those in MFD-I. This can be attributed to the particular arrangement of pore grains in MFD-II, where the smaller grains in the middle of the pore body (Figure 3.2), may have reduced the initial fluorescent particle cloud velocity more quickly. The impact of flow velocity on ATDC stabilization in MFDs indicates that the higher flow velocity causes higher disparity in ATDC values at the two upstream cross-sections in both the devices (Figure 3.5). This effect of flow velocity was again consistent with observations made by Acharya et al. (Acharya et al. 2007) where larger variation in transverse dispersion coefficients were observed at larger Peclet numbers (i.e. higher flow velocities).

Additionally, some of the experimental limitations, such as the pulse flow generated by the syringe pump (kd Scientific, Model 200 Series) used in this study, may have also contributed to the disparity in the ATDC values near the tracer inlet point. As a result, the longer error bars corresponding to the profiles at 4.9 and 9.8 mm as compare to downstream profiles were observed (Figure 3.5). The asymmetric intensity distribution profiles in Figure 3.3 further support the effect of pulse flow.

While dispersivity is generally considered as an intrinsic property of the porous medium, different longitudinal (Hornberger et al. 1992; Dong et al. 2002) and transverse (Long and Ford 2009) dispersivity values have been reported for the same porous media based on the dispersant used. In their colloidal transport study in micromodels, Auset and Keller (Auset and Keller 2004) have emphasized the dependence of dispersivity on the size of the dispersant in addition to properties of the porous medium. Results from this study also show three different values of apparent transverse dispersivity for similar porous media under three different experimental conditions (with bacteria, with non-motile bacteria, and without bacteria) when
the same dispersant (FITC-dextran) was used. Therefore, we suggest that dispersivity may be considered as a system-specific parameter, incorporating effects of the porous medium and dispersant, and characteristics of the transporting fluid.

**3.2.1. Mixing enhancement Index:** A mixing enhancement index (MEI) was defined to quantify the extent of mixing enhancement and was quantified in terms of the ratio of the effective transverse dispersion coefficient values in experiments with and without motile bacteria:

\[
\text{Mixing enhancement index (MEI)} = \frac{\langle (D_y)_{eff} \rangle \text{with bacteria}}{\langle (D_y)_{eff} \rangle \text{no bacteria}}
\]

The effect of flow velocity on the MEI is shown in Figure 3.7. For MFD-I and MFD-II, MEI shows an increasing trend initially and finally decreases at the highest flow velocity used in this study. Mixing enhancement in the presence of motile bacteria in these devices may be attributed to the free swimming of bacteria across flow streamlines creating turbulence in their micro-environment. A common understanding may suggest that increasing Darcy velocity should adversely affect bacterial random motility and thereby the contaminant mixing index. This belief would be further strengthened by the fact that higher flow velocities will cause greater shear rates on motile bacteria in porous MFDs. However, Marcos and Stocker (Marcos and Stocker 2006) showed in their study of the effect of shear flow on the motility of the marine bacterium *Pseudomonas haloplanktis*, a bacterium with similar shape, size, and swimming properties to *E. Coli* HCB 33, that bacteria are capable of overcoming vortices of moderate strength. Experimental results of Marcos and Stocker (Marcos and Stocker 2006) showed bacteria crossing flow streamlines under moderate shear flow conditions as well as aligning themselves across the flow stream lines when vortices were switched from low to moderate strength. Lanning et al. (Lanning et al. 2008) have also reported similar transverse movement of *E. Coli* HCB1 across flow stream lines at Darcy velocities greater than those used in this study (~1
mm/sec) as a result of chemotactic migration. Thus, it can be argued that the flow velocity range of 0.11 mm/s to 0.44 mm/s used in this study may cause low to moderate shear flow conditions and thereby result in an increase in MEI (Figure 3.7). The decrease in the MEI values in MFD-I and MFD-II at the highest Darcy velocity (0.88 mm/s) suggests that at stronger shear flow rates, bacteria not only follow the flow streamline but also align their body axis with the flow lines (Marcos and Stocker 2006).

In contrast to the other two devices, the observed MEI in MFD-III remained approximately constant for all Darcy velocities tested. The reason for this difference may be attributed to the pore throat spacing of MFD-III, because all other features of this device are similar to MFD-I. Typical mean bacterial run lengths for *E. coli* HCB1, a bacteria similar to *E. coli* HCB33 used in this study, are ~ 28 µm (calculated based on the values reported by Lewus and Ford [44]), which is comparable to the pore throat size in MFD-III (32 µm). However, the pore throat size in MFD-I (50 µm) is significantly larger and would cause less interfere with bacterial free swimming paths as compared to MFD-III. Therefore, we believe that the ratio of pore throat spacing to the bacterial run length may be a critical parameter for enhanced mixing, though this speculation should be verified with single cell experiments. Comparing across the three devices, MFD-II resulted in the highest MEIs for almost all flow velocities, which may be attributed to its specific pore geometry; the smaller grain in the middle of the pore, which was not present in the other two pore geometries, may have acted as an additional agent of mixing in this device.

Results from this study contribute to a better understanding of mass transfer mechanisms in porous media and provide a framework for incorporating mixing enhancement due to bacterial motility in bioremediation models. In most bioremediation studies at the
laboratory and field scales, contaminant transport is predicted based on tracer tests as a surrogate for contaminants that do not involve bacteria (Long and Ford 2009). Such contaminant transport predictions may be erroneous as results from this study show that motile bacteria may provide significant contaminant mixing in porous media. Incorporating contaminant mixing due to bacterial motility in field scale bioremediation strategies such as monitored natural attenuation may be useful for more accurate predictions of remediation time frames. Further research may look beyond the effect of bacterial random motility on contaminant mixing in porous media to include other motility mechanisms such as chemotaxis, which is more likely to prevail and may dominate mixing enhancement in certain bioremediation scenarios.
Figure 3.7: Effect of flow velocity on mixing enhancement due to bacterial random motility in different MFDs. Error bars indicate standard errors.
4. Abstract

Low permeability regions such as clay lenses are difficult to remediate using conventional treatment methods. Bacterial chemotaxis (directed migration toward a contaminant source), may be helpful in enhancing bioremediation of such contaminated sites. This study experimentally simulates a two-dimensional dual-permeability groundwater contamination scenario using a microfluidic device (MFD-IV) and evaluates transverse chemotactic migration (in response to chemoattractant DL-aspartic acid) of bacteria (*Escherichia coli* (*E. coli*) HCB33) from high to low permeability regions. Bacterial chemotaxis was quantified in terms of change in total bacterial counts in pore throats in low permeability region. A significant change in total bacterial counts, ranging from 1.09 to 1.74 times, was observed in low permeability region in experiments under chemotactic conditions. While, experiments under non-chemotactic conditions did not show any such increment in the total bacterial counts. A large increase in bacterial counts in the pore throats just outside the low permeability region was also observed in chemotaxis experiments. Effect of flow velocity was also evaluated and the bacterial chemotactic response was observed to decrease linearly with increase in flow velocity and the effect was observed to offset by the advective flow at the highest flow velocity (Darcy velocity = 0.22 mm/s) used during this study.

4.1. Introduction

Low permeability regions in the subsurface are of great concern for remediation of groundwater contaminants, including non-aqueous phase liquids (NAPLs) which may remain trapped in these regions. Conventional treatment technologies such as pump-and-treat are ineffective at
completely removing these trapped contaminants from low permeability regions; over time they may diffuse out into high permeability regions (Liu and Ball 2002; Olson et al. 2006) thereby acting as long term sources of groundwater contaminants. *In situ* bioremediation has been suggested as one of the treatment methods for effective and economical removal of contaminants from these sites (Scow and Hicks 2005; Jørgensen et al. 2009).

Many soil-inhabiting motile bacteria are chemotactic toward various groundwater pollutants and are capable of transforming them into non-toxic compounds (Pandey and Jain 2002; Parales and Harwood 2002). Chemotaxis, the ability of bacteria to sense and respond to chemical gradients and swim toward optimal concentrations of potential substrates, enables bacteria to move into niches that are best suited for their growth and survival (Pandey and Jain 2002; Olson et al. 2004; Ford and Harvey 2007). The importance of chemotaxis in bioremediation of recalcitrant xenobiotics has been discussed in the literature (Pandey and Jain 2002; Parales and Harwood 2002; Singh and Olson 2008). Chemotaxis can potentially improve *in situ* bioremediation of groundwater by: (1) increasing the rate of contaminant consumption by positioning bacteria in favorable conditions for metabolism; (2) increasing the number of metabolically active bacteria in the area surrounding a NAPL thereby further enhancing the aqueous chemical consumption rate; (3) reducing aqueous concentrations in the surrounding bulk media, thereby increasing the chemical diffusion gradient and increasing the dissolution rate of the NAPLs; and (4) limiting the toxic effects of aqueous chemical concentrations to bacteria by enabling them to move away from higher, toxic concentrations (Baena-Ruano et al. 2006; Singh and Olson 2008).

Sharp chemical gradients are ubiquitous in dual permeability regions of groundwater aquifers where chemicals trapped in low permeability regions diffuse into more highly permeable zones. These regions provide ideal environments wherein chemotactic migration
may be harnessed, if appropriately engineered, for enhancing bioremediation of contaminants in low permeability regions. Olson et al. (2006) performed mathematical simulations in a two dimensional heterogeneous porous medium with contaminant trapped in low permeability zone, and concluded that chemotactic bacteria preferentially swim toward and accumulate in low permeability regions leading to significant enhancement in trichloroethylene removal. Interfaces between two different permeability regions are of critical importance in determining the extent of the bacterial response to trapped contaminants. As contaminants diffuse out from low permeability regions, sharp chemical gradients form as water is flushed due to differences in flow rates through the two regions. These chemical gradients may elicit chemotactic bacterial accumulation at these interfaces. Additionally, bacteria responding chemotactically from high permeability regions to low permeability regions may experience impedances in their increased run lengths due to factors such as Knudsen diffusion (Olson et al. 2005, 2006), resulting in further accumulation of bacteria at the interfaces. The increased concentration of bacteria at interfaces may in turn result in an increase in the rate of chemical consumption, creating an even steeper chemical gradient. Therefore bacterial behavior near chemical gradients may play a vital role in the bioremediation of low permeability regions by increasing the rate of contaminant diffusion out of the low permeability region and further enhancing the chemotactic response of bacteria in a positive feedback loop (Olson et al. 2006).

Many researchers have endorsed the significance of chemotactic bacterial transport for enhancement of in situ groundwater bioremediation (van der Meer et al. 1987; Bosma et al. 1988; Harvey and Garabedian 1991; Olson et al. 2004, 2006; Ford and Harvey 2007; Long and Hilpert 2007; Singh and Olson 2008; Long and Ford 2009). Chemotactic bacterial migration in chemical gradients in aqueous environments and under no flow conditions has been extensively investigated in the literature (Berg and Brown 1972; Adler et al. 1973; Harwood et al. 1990;
Marx and Aitken 1999; Parales and Harwood 2002; Law and Aitken 2003; Olson et al. 2004; Hilpert 2005; Lanning et al. 2008) with various groundwater pollutants such as naphthalene, toluene, trichloroethylene etc (Marx and Aitken 1999; Parales and Harwood 2002; Law and Aitken 2003; Olson et al. 2004). The presence of porous media in groundwater aquifers may impede bacterial chemotactic migration due to Knudsen diffusion (reduced bacterial path lengths due to collision with the pore walls) (Olson et al. 2006). Therefore, considering the effects of porous media and advective flow on bacterial migration is critical in determining the conditions under which chemotaxis significantly affects transport. The effect of porous media on bacterial chemotactic migration has been investigated using packed column experiments (Pedit et al. 2002; Olson et al. 2004) and mathematical simulations (Pedit et al. 2002; Olson et al. 2004; Hilpert 2005; Olson et al. 2006; Long and Hilpert 2007; Long and Ford 2009). Recent studies have focused on chemotactic bacterial migration under advective flow conditions in bulk aqueous (Lanning et al. 2008) and porous (Long and Ford 2009) environments. These studies have used microfluidic devices that allow formation of two impinging streams of bacterial and attractant suspensions flowing side by side, and report chemotactic bacterial migration transverse to the direction of flow. This study extends previous work to a more relevant groundwater contamination scenario by studying bacterial transverse migration in a dual permeability porous microfluidic device.

The goal of this work is to experimentally simulated a 2D heterogeneous aquifer with contaminant trapped in a low-permeability lens, and to study the effect of chemotaxis on bacterial migration toward the low permeability region. We employ a novel two dimensional bi-layer dual-permeability porous microfluidic device that simulates a contaminated low permeability region surrounded by a high permeability region. Transverse migration of *Escherichia Coli* HCB33 was studied under the influence of the chemoattractant DL-aspartic acid.
The enhancement of bacterial concentrations in the low permeability region was quantified in terms of the incremental increase in total bacterial counts in pore throats at cross-sections downstream from the MFD inlet with respect to bacterial counts at a base cross-section near to the inlet. Results were compared with control experiments under non-chemotactic conditions. The effect of Darcy flow velocity on chemotactic migration toward the low permeability region was also studied. To the best of our understanding, this paper presents the first experimental validation of chemotactic bacterial accumulation in low-permeability regions of a heterogeneous porous medium containing a contaminant source, and therefore provides an important step toward the application of bacterial chemotaxis for remediation of clay lenses.

4.2. Materials and Methods

4.2.1. Cell culture and chemicals preparation

*Escherichia coli* HCB33 (wild type strain) cells were obtained from Dr. Min Jun Kim of Drexel University. Cells from a -80 °C frozen glycerol stock were grown on Luria broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter of DI water) in a shaking incubator @ 200 rpm (VWR Incubating Orbital Shaker) at 33 °C for 4 h. Cells were harvested in their exponential growth phase at an optical density at 590 nm (OD<sub>590</sub>) of ~ 0.8-1.0 (Spectronic® 20 GENESYS™ Spectrophotometer). Cells were washed with 10% random motility buffer (RMB) (11.2 g K<sub>2</sub>HPO<sub>4</sub>, 4.8 g KH<sub>2</sub>PO<sub>4</sub>, and 0.029 g EDTA per liter of DI water; pH ~ 7.0) by centrifugation and were concentrated to approximately 20 times the original concentration by resuspending in 10% RMB for plug assay experiments, described in the following section. For chemotaxis experiments in the MFDs, the cells harvested from the exponential growth phase were diluted to a final concentration of OD<sub>590</sub> = 0.60 (~1.5x10<sup>9</sup> cells/ ml) after centrifugation and washing with 10% RMB. DL-aspartic acid, a chemoattractant for *E. coli* HCB33, was used as a contaminant
surrogate in this study; a stock solution of 50 mM (6.66 g/L) was prepared in 10% RMB and diluted to the desired concentration for each specific experiment.

4.2.2. Plug assay experiments

Plug assay experiments, commonly used to verify bacterial chemotactic response toward various environmental pollutants (Yu and Alam 1997; Parales et al. 2000; Olson et al. 2004), were used in this study to determine the DL-aspartic acid concentration that elicits the optimum chemotactic response for *E. coli* HBC33. Full details of plug assay experiments are described previously (Singh and Olson 2010). Briefly, 0.1 g of low-melting-temperature agarose (NuSieve GTG Agarose, FMC Bioproducts, Rockland, Maine) was added to 0.5 mL of different concentrations of DL-aspartic acid buffer solution in a microcentrifuge tube and melted at 80 °C in a water bath. A small droplet of the melted DL-aspartic acid-containing agarose was placed on the top of a microscopic slide with two thin plastic strips placed on either side of the droplet, approximately 2 cm apart. A cover slip, supported by the plastic strips at the ends, was placed on top of the plug, resulting in the formation of a chamber surrounding the plug droplet. The prepared bacterial suspension was flooded into the chamber surrounding the plug, creating a system representative of a NAPL droplet dissolving in ground water. Phase contrast microscopy was used to investigate the tactic response of *E. coli* HCB33 toward DL-aspartic acid. Images were captured using a CCD camera (AxioCam, Carl Zeiss) mounted on a stereoscope (Zeiss Stereo Discovery V8). The plug assay experiment was repeated with different concentrations of DL-aspartic acid by diluting the 50 mM stock solution to find the optimum chemoattractant concentration for *E. Coli* HCB33 for subsequent chemotaxis experiments in MFDs.

4.2.3. Design and fabrication of microfluidic devices (MFDs)

The microfluidic device used in this study was designed to simulate a contaminated 2D groundwater aquifer, containing a region of low permeability surrounded on both sides by
regions of high permeability. The microfluidic device with this design will be termed as MFD-IV from here onward. This design is aimed to simulate a scenario in which a leaking underground storage tank is continually contaminating an aquitard. The design and fabrication of the device was similar to that described in previous chapter (Section 3.3.1), modified with a dual permeability pore design. The modified schematic of the device is presented in Figure A.1 (Appendix-A). The physical design of the device consists of a porous channel, simulating a 2D groundwater aquifer (~ 0.022 mm thickness), overlaid by a small non-porous channel that act as source of contaminant/attractant injecting into the low permeability region of the porous bottom channel. Standard photolithography and soft lithography techniques were used for fabrication of the device (Duffy et al. 1998; Whitesides and Stroock 2001) and specific details are described in previous chapter (Section 3.3.1).

Details of the pore arrangement of the device (MFD-IV) used in this study are shown in Figure 4.1. Figure 4.1a shows the transverse pore arrangement in a small longitudinal cross section of the dual-permeability heterogeneous porous medium, which consists of a 1.75-mm-wide low permeability region sandwiched between two 2.10-mm-wide layers of high permeability regions, which extends 40 mm longitudinally. The mask design of the high permeability regions consists of a regular pattern of uniform dark circles of diameter 0.300 mm (representing soil grains) spaced 0.350 mm apart and therefore leaving a pore throat spacing of 0.050 mm between them (Figure 4.1b, Table 4.1). The design of the low permeability layer consists of a similar pattern of dark circles, along with one smaller circle of 0.100 mm diameter right in the middle of the pore space resulting from the four larger circles (Figure 4.1b, Table 4.1). The other properties of the porous media are given in Table 4.1. The Kozeny-Carman equation (McCabe et al. 2005) was employed to estimate the permeabilities ($k$) of the two regions using eq 3.1:
4.2.4. Microfluidic device (MFD) setup and operation for chemotaxis experiments

A multiple loading syringe pump (kd Scientific, Model 200 Series) was used for injecting a chemotactic bacterial suspension into the porous bottom channel of the MFD-IV and a chemoattractant (20 mM DL-aspartic acid) solution into the top channel that drips into the middle of the low permeability porous region. The top channel was connected to a 1-mL syringe (BD, internal diameter 4.77mm) and the bottom channel was connected to a 10-mL syringe (BD, internal diameter 14.50 mm) to acquire the desired volumetric flow rate ratio of 9.2 : 1 from the bottom : top channels. Polyethylene tubing (INTRAMEDIC® Polyethylene Tubing, Clay Adams®) was used to attach the punched inlets of the channels to their respective syringes via syringe needles (BD 21G1). Experiments were performed at average Darcy velocities (calculated by dividing total volumetric flow rates through both ports by the total cross-sectional area of the flow channel) of 0.055, 0.11, and 0.22 mm/s, a range chosen from similar groundwater studies in micromodels (Willingham et al. 2008; Long and Ford 2009). Equilibrium flow conditions were ensured by allowing at least two pore volumes of fluid to flow through the device before chemotactic bacterial accumulation data were acquired. For comparison, control experiments were run under non-chemotactic conditions in which the chemottractant (5 mM DL-aspartic acid) solution was replaced with 10% RMB in the top channel.

4.2.5. Data acquisition and analysis

In order to quantify bacterial chemotaxis, three cross-sections were chosen transverse to the flow direction at 4.9, 9.8 and 19.6 mm downstream from the chemoattractant injection point into the porous bottom channel. Images were captured in each transverse pore throat of the three cross-sections under a phase contrast microscope at 63X magnification (Figure 4.1b and c). The choice of pore throats as opposed to pore bodies as sampling locations for bacterial counts was determined both conceptually and by experimental limitations. Upon careful consideration
Figure 4.1: Schematic showing the details of the pore arrangement and sampling (imaging) area in the microfluidic device (MFD-IV). (a) The transverse pore arrangement simulates a two dimensional porous medium in which a low permeability layer of aquifer is sandwiched between two high permeability layers. (b) A zoomed-in view of the pore geometries of the high permeability zone (repeated pattern of uniform size grains) and low permeability zone (repeated pattern of two different sized grains). Also shown are the transverse pore throat areas where images were captured. (c) A representative microscopic image in the transverse pore throat with bacterial counting area highlighted in the middle of the pore throat. The transverse length of the counting area is 0.10 mm. Other properties of the device are given in the Table 4.1 and complete details of the overall physical geometry and the operation of the device are discussed in the previous chapter 3, section 3.3.1.

Table 4.1: Hydrogeologic properties of microfluidic device (MFD-IV).

<table>
<thead>
<tr>
<th>Zone type</th>
<th>Pore geometry type</th>
<th>Grain(s) size, $D_p$ (mm)</th>
<th>Pore throat spacing (mm)</th>
<th>Porosity, $\varepsilon$ (%)</th>
<th>Permeability (mm$^2$)</th>
<th>Apparent transverse dispersivity ($\alpha_{app}$) (mm)</th>
<th>$h^{**}$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low permeability zone</td>
<td>Repeated pattern of non-uniform grains</td>
<td>0.30, 0.10</td>
<td>0.050</td>
<td>35.9</td>
<td>$0.44 \times 10^{-4}$</td>
<td>0.003</td>
<td>0.56±0.04</td>
</tr>
<tr>
<td>High permeability zone</td>
<td>Repeated pattern of uniform grains</td>
<td>0.30</td>
<td>0.050</td>
<td>42.3</td>
<td>$1.36 \times 10^{-4}$</td>
<td>0.005</td>
<td>-</td>
</tr>
</tbody>
</table>

*Value is calculated based on the geometric mean grain size of the pore designs shown in Figure 4.1(b).

** Value represents an average of triplicate tracer transport experiments in chapter 3.
of the velocity distribution in the pore throats and pore bodies of the MFD-IV, it was apparent that for a given Darcy velocity, flow lines in longitudinal pore throats and pore bodies would experience relatively greater velocity in comparison to in transverse pore throats, where relatively moderate flow conditions exist. Due to this intra-pore velocity distribution, the local chemical gradient may prevail between transverse pore throats and pore bodies, which may trigger bacterial chemotactic migration in transverse pore throats. Also, pore body designs differ between the two permeability regions of the device due to the presence of an extra grain in the middle of the pore body in the low permeability region (note the difference in the two images in Figure 4.1b), thereby making it difficult to compare the bacterial count data in pore bodies from two different permeability regions. Additionally, bacterial counting was itself difficult in the images taken within the pore bodies due to bacterial flow with the stream lines under advective flow conditions.

For quantification purposes, bacteria were enumerated in the middle area (width 0.1 mm) of the pore throat as shown in Figure 4.1(c) to avoid any interference from the flow in the pore bodies. A graphic user interface (GUI) was designed in MATLAB R2010b (MathWorks®) for counting bacteria to obtain consistency both in counting and in area selection. Bacterial count data in the pore throats at the extreme outer ends of the device at each cross-section were not included in the analysis because a large number of bacteria were attached to the side walls of the terminal pores.

Results from triplicate experiments performed under both chemotactic and non-chemotactic conditions were analyzed to study bacterial chemotactic migration in dual permeability porous MFD-IV. Base bacterial counts were taken in transverse pore throats at a cross-section 4.9 mm downstream from the injection point. Bacterial counts in corresponding pore throats at further downstream cross-sections, i.e. 9.8 mm and 19.6 mm, were also measured. The change in
bacterial counts in the respective transverse pore throats at downstream sections with respect to base bacterial counts at the upstream cross-section were analyzed and used as an indicator of bacterial chemotaxis. The relative bacterial count profiles at cross-sections 9.8 mm and 19.6 mm downstream were plotted. Due to symmetry of the device only one half of the device (transverse to the flow direction) (i.e. y≥0) was chosen for analysis.

4.2.6. Transverse dispersion of DL-aspartic acid (Dchy)

The two-dimensional advection-dispersion equation (Eq. 3.2) used for tracer transport in porous media was employed for chemoattractant (DL-aspartic acid) transport prediction in this study. The experimental conditions used for chemoattractant transport in this study were similar to that for tracer transport in chapter 3. Therefore, eq. 3.4 was modified for concentration prediction by replacing fluorescent intensity term (I) with chemoattractant concentration (C) term:

\[
\frac{C}{C_0} = \frac{1}{2} \left( \text{erf} \left( \frac{h_c - y}{2(D_{chy})_{eff}^{X/v_f}} \right) + \text{erf} \left( \frac{h_c + y}{2(D_{chy})_{eff}^{X/v_f}} \right) \right)
\]

where, \( C \) is local chemoattractant concentration, \( C_0 \) is the maximum concentration of DL-aspartic acid, \( h_c \) is with of attractant plume near the inlet which is assumed to have constant concentration for the entire width, \( x \) and \( y \) are longitudinal and transverse directions, respectively, \( v_f \) is linear velocity of water through pores and \((D_{chy})_{eff}\) is effective transverse dispersion coefficient of DL-aspartic acid in porous media and is given by:

\[
(D_{chy})_{eff} = (D_{co})_{eff} + \alpha_{app} v_f
\]

where, \((D_{co})_{eff}\) is effective diffusion coefficient and \(\alpha_{app}\) is apparent transverse dispersivity of the porous medium. Both, \((D_{co})_{eff}\) and \(\alpha_{app}\) are lumped parameters that account for the effect of the presence of bacteria and porous media in the system. The effective diffusion coefficient of DL-aspartic acid can be given by (Sherwood et al. 2003; Olson et al. 2005)
\[(D_{c0})_{eff} = \frac{\varepsilon}{\tau} D_{c0} \] 4.3

where, \(\varepsilon\) and \(\tau\) are porosity and tortuosity of the porous medium and \(D_{c0}\) is the molecular diffusion coefficient of the DL-aspartic acid, the value of which is \(D_0=8.6 \times 10^{-4}\) mm\(^2\)/s (Lanning et al. 2008). The tortuosity term incorporates the effects of the available pore space and the transport mechanism of the tracers (Olson et al. 2005) and an assumed value of 2 was used in this study based on values used in similar groundwater studies (Acharya et al. 2007; Long and Ford 2009).

Eq. 4.1 is used to predict the DL-aspartic acid concentration profiles at different cross-sections by substituting the parameter values calculated from eqs. 4.2 and 4.3. The porosity \(\varepsilon\) values for the different pore geometries are reported in Table 4.1. Apparent transverse dispersivity values for the two pore geometries were taken from the values obtained in chapter 3 under similar experimental conditions, i.e. with motile bacteria in the system and are summarized in Table 4.1. The width of the constant attractant concentration, \(h_c\), was calculated from tracer tests performed previous chapter 3, where porous media geometry similar to the low permeability region was used and ‘\(h_c\)’ was measured immediately downstream \((x=1.05\) mm\) from the point of injection of the chemical into the porous bottom channel. The value of ‘\(h_c\)’ is reported in Table 4.1 and represents the average of triplicate experiments along with standard deviation. Due to symmetry of the device, the concentration profiles were cut in half at \(y=0\), and only half of the porous channel region \((y\geq0)\) was chosen for analysis.

4.3. Results and Discussion

4.3.1. Plug assay experiments

Plug assay experiments were performed for the following concentrations of DL-aspartic acid: 0mM, 10 mM, 15mM, 20 mM, and 25 mM. Bacterial accumulation bands surrounding the plug were observed as an indicator of \textit{E. Coli} HBC33 chemotaxis toward DL-aspartic acid at various
time intervals. It was observed that for 0, 10, and 15 mM DL-aspartic acid concentrations in the plug, there was no significant bacterial accumulation around the plug. However, increasing the DL-aspartic acid concentration in the plug to 20 and 25 mM elicited bacterial accumulation bands.

Figure 4.2 shows images of plug assay experiments in chemotactic (20 mM DL-aspartic acid) (upper row) and control (0 mM DL-aspartic acid) (lower row) conditions at different time intervals. It can be observed that at the start of the experiments (t= 0 min), the bacterial concentrations (represented by the grey intensity) around the plug are evenly distributed in the in both chemotactic (Figure 4.2a) and control (Figure 4.2f) experiments. Under chemotactic conditions, DL-aspartic acid diffuses out from the plug into the surrounding bacterial chamber as time progresses, and an intense ring of bacterial accumulation can be observed around the plug from 2 min to 10 min (Figure 4.2b through 4.2e) after the onset of the experiment. However, no such accumulation was seen in control experiments (Figure 4.2g through 4.2j). This observation verifies the chemotactic response of E. coli. HCB33 to 20 mM DL-aspartic acid, and therefore a similar concentration of DL-aspartic acid was used as the chemoattractant in subsequent chemotaxis experiments in the MFD-IV.

4.3.2. Transverse chemotactic migration of bacteria in MFD-IV

Bacterial count profiles at cross-sections 9.8 mm and 19.6 mm downstream from the attractant injection point in experiments run at a Darcy velocity of 0.11mm/s were plotted as relative bacterial counts, i.e. the ratio of the bacterial counts at any specific pore throat at these cross-sections to the bacterial counts in the corresponding pore throat at the base cross-section (4.9 mm from the injection point), under both chemotactic and non-chemotactic conditions (Figure 4.3). Normalized attractant concentrations (with respect to maximum value) at cross-sections
Figure 4.2: Results from Plug assay experiment to determine the optimal chemoattractant concentration of L-aspartic acid for *Escherichia coli* HCB33. Images in the upper row correspond to a representative chemotactic experiment (0.20 mM aspartic acid as chemoattractant in plug) and lower row images show results from a representative control experiment (no chemoattractant in plug) at various time intervals. The horizontal arrow in each image indicates the cross-section along which the grey intensity is analyzed and is accompanied by the intensity profile that represents bacterial concentration distribution surrounding the plug.
4.9, 9.8, and 19.6 mm downstream from the injection point are also plotted in Figure 4.3. The hump in the predicted attractant concentration profiles at the interface between the two permeability regions is due to the different transverse dispersivity and porosity values for the two porous media. It can be observed that under non-chemotactic conditions, relative bacterial counts in the low permeability region are less than 1, with an outward increasing trend in the relative bacterial counts profiles at both the cross-sections. This profile trend may be attributed to the dilution effect resulting from the additional flow injection of bacteria-free solution from the top channel into the low-permeability bottom channel; this flow may also push bacteria outward from the center of the device. The slight increase in bacterial counts in pore throats at the interface of the two permeability regions may be a result of diffusion limitation from the high to low permeability regions. Also, the physical difference in the pore geometries (i.e. the additional grain in the middle of the pore in the low permeability region) may also contribute to the bacterial accumulation in the pore throat at the interface. In chemotaxis experiments the relative bacterial count values in the low permeability region were around 1 and 1.35 at cross-section 9.8 and 19.6 mm downstream, respectively, compared with approximately 0.6 at both the downstream cross-sections under non-chemotactic conditions (Figure 4.3). Bacterial count profiles at both the downstream cross-sections show a large increase in bacterial counts in pore throats just outside of the low permeability region with a corresponding decrease in bacterial counts in pore throats at further transverse distances in the MFD-IV. This effect was more pronounced at the 9.8 mm cross-section. It can be seen that the large bacterial accumulation band observed in the 9.8 mm cross section approximately coincides with the predicted attractant concentration tail at the same cross-section (Figure 4.3). Though the relative bacterial count in the low permeability region at this cross-section remained approximately 1, comparing with non-chemotactic conditions, it can be considered a significant increase given the
Figure 4.3: Representative relative bacterial count profiles in chemotactic (CHTX) and non-chemotactic (CTRL) experiments along with normalized attractant concentration profiles (ATTR, in chemotactic experiments only) in transverse pore throats in experiments at Darcy velocity 0.11 mm/s. Bacterial counts in pore throats at cross-sections 10 mm and 20 mm are relative to the bacterial count in the corresponding pore throats at a base cross-section 5 mm downstream from the attractant/buffer inlet into the porous channel. Attractant concentration profiles were normalized with respect to the maximum concentration.
presumed dilution due to flow from the top channel. Upon moving further downstream to the 19.6 mm cross-section, the bacterial count peak in the pore throat immediately outside the low permeability region decreased, with a corresponding increase in the bacterial count in the low permeability zone (Figure 4.3). This shift, which represents further accumulation of bacteria in the low permeability zone, may be attributed to the fact that a steeper chemical gradient exists in the low permeability region toward the centerline of the device, which may have attracted more bacteria from the high to the low permeability region. These observations clearly indicate chemotactic migration of E. Coli HBC33 from the high to the low permeability regions of the device.

### 4.3.3. Bacterial accumulation in low permeability region

In order to quantify chemotactic bacterial migration toward the low permeability region of the MFD-IV, the total number of bacteria were counted in the middle six transverse pore throats of the MFD-IV (four pore throats in the low permeability region plus two adjacent pore throats on either side of the low permeability region where maximum bacterial accumulation is expected) at three cross-sections: 4.9 mm, 9.8 mm, and 19.6 mm downstream from the injection point. Similar to the previously described analysis, chemotaxis toward the low permeability region was quantified in terms of the relative increase in total bacterial counts in the low permeability region at cross-sections 9.8 mm and 19.6 mm downstream from the inlet with respect to the 4.9 mm cross-section. Relative total bacterial counts in the low permeability region at cross-sections 9.8 mm and 19.6 mm under chemotactic and non-chemotactic conditions are reported in Table 4.2 at three different Darcy velocities. Each data point represents the average value of triplicate experiments along with standard deviation. Under chemotactic conditions a significant increase in the relative bacterial count at cross-section 9.8 mm was observed for Darcy velocities 0.055 mm/s and 0.11 mm/s. A further increase in the relative bacterial counts was observed for both
**Table 4.2:** Change in bacterial counts in low permeability zone at various cross-sections under chemotactic and non-chemotactic conditions. Average relative bacterial counts and standard deviation are reported for triplicate experiments.

<table>
<thead>
<tr>
<th>Experimental Darcy velocity (mm/s)</th>
<th>Relative change in bacterial count with respect to the base (5 mm) cross-section</th>
<th>Chemotactic conditions</th>
<th>Non-chemotactic conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 mm</td>
<td>20 mm</td>
</tr>
<tr>
<td>0.055</td>
<td></td>
<td>1.27±0.11</td>
<td>1.74±0.13</td>
</tr>
<tr>
<td>0.110</td>
<td></td>
<td>1.09±0.15</td>
<td>1.32±0.20</td>
</tr>
<tr>
<td>0.220</td>
<td></td>
<td>0.89±0.15</td>
<td>0.75±0.21</td>
</tr>
</tbody>
</table>
velocities at the cross section 19.6 mm downstream from the injection point. In contrast, under non-chemotactic conditions at similar Darcy flow velocities, relative bacterial counts were observed to decrease with distance at cross-sections downstream from the inlet point. This decrease in bacterial count may be attributed to the dilution due to buffer injection from the top channel into the low permeability region in the MFD-IV.

On increasing the Darcy velocity to 0.22 mm/s, the relative total bacterial count was observed to decrease at both downstream cross-sections (Table 4.2) for both chemotactic and non-chemotactic experiments, which indicates that advective flow was a dominant factor at high flow rate that may have offset bacterial chemotaxis. Under non-chemotactic conditions, the results were not significantly different than the other two velocities. A marginally higher relative bacterial count was observed under chemotactic conditions at a Darcy velocity of 0.22 mm/s (row 3, Table 4.2). This observation indicates that even at this flow velocity bacteria may still have migrated chemotactically against the dilution, however, other factors such as shortened response time and greater shear at high flow velocity may have contributed to the comparatively lower relative total bacterial count.

Under chemotactic conditions, relative total bacterial count shows a linear decreasing trend with respect to increasing Darcy velocity at both downstream cross-sections (Table 4.2). However, no definite trend was observed under non-chemotactic conditions where the relative total bacterial count remained nearly unchanged at both cross-sections for each velocity. Under non-chemotactic conditions, slightly lower relative total bacterial counts were observed at the 19.6 mm cross-section, compared with the 9.8 mm cross-section, at all three velocities tested. These observations further verify that dilution with bacteria-free buffer stream injected into the low permeability region was the main factor for low relative bacterial counts in this case because (1) the ratio of the flow in both the ports also remained constant, i.e. 9.2:1, during
experiments for each Darcy flow velocity, and (2) the dilution effect is expected to be more pronounced further downstream cross-section.

The base cross-section was chosen 4.9 mm downstream from the attractant injection point because of optical interference from the top channel near the inlet. The predicted attractant concentration profile outside the low permeability region was negligible at cross-section 4.9 mm, therefore chemotactic migration into the low permeability region was unlikely at that cross-section. Additionally, to minimize chemotactic migration toward the low permeability region at 4.9 mm, if there was any, images in the pore throats at the base cross-section were taken immediately after the flow was established (less than 5 min) in the device whereas other downstream cross-sections were imaged after steady state flow conditions were achieved (15 to 20 min).

Transverse migration of bacteria from high permeability regions to low permeability regions in response to chemical gradients has been theoretically predicted by (Olson et al. 2006), but is confirmed experimentally in this study. Low permeability regions, such as aquitards, are difficult to remediate, as traces of contaminants remain trapped in them even after conventional treatment has removed the more accessible contamination. Application of chemotactic migration for remediation of these sites may be extremely useful in removal of contaminants. (Heald and Jenkins 1994) reported a two-fold increase in trichloroethylene removal by doubling the biomass concentration. Results from this study are encouraging, showing nearly a 2-fold increase in bacterial concentration in low permeability regions, which may prove significant in the bioremediation of contaminated aquitards.
5. Abstract

The goal of the present chapter is to better elucidate the distribution of viable bacteria in chemical gradients and to evaluate the toxic effect of high concentrations of contaminants on contaminant-degrading bacteria under prolonged exposure. Accumulations of viable *Pseudomonas putida* F1 (*P. putida* F1) cells were observed surrounding trichloroethylene (TCE) - containing plugs. Results from this work indicate that *P. putida* F1 immediately adjacent to a TCE source become non-viable, while cells accumulating further away use chemotaxis to migrate toward regions with optimal chemical concentrations in the form of concentrated bacterial bands. A method was developed to test the toxicity of model contaminant stressors, TCE and toluene, to *P. putida* F1; data obtained from toxicity experiments were fit to linear and exponential bacterial viability-decay models. Toxicity of TCE to *P. putida* F1 was best described with an exponential viability-decay model, with a viability-decay constant $k_{TCE} = 0.025$ h$^{-4.95}$ ($r^2 = 0.965$). Toluene toxicity showed a marginally better fit to the linear viability-decay model ($r^2 = 0.976$), with a viability-decay constant $k_{toluene} = 0.208$ h$^{-1}$. Best-fit model parameters obtained for both TCE and toluene were used to predict bacterial viability in toxicity experiments with higher contaminant concentrations and matched well with experimental data. Results from the present study can be used to predict bacterial accumulation and viability near nonaqueous-phase liquid (NAPL) sources in groundwater, and may be helpful in designing bioremediation strategies for sites contaminated with residual NAPLs.

*Most of the material contained in this chapter is published in *Environ Toxicol Chem.* 2010 Jan;29(1):56-63*
5.1. Introduction

Nonaqueous-phase liquids (NAPLs) remain challenging as long-term sources of ground water pollution due to their slow dissolution rates and strong adhesion to soil particles in low-permeability regions (Liu and Ball 2002). Conventional treatment technologies, such as pump and treat, are not only economically infeasible but also ineffective at completely removing NAPLs (Olson et al. 2004). Various soil-inhabiting microbes are capable of degrading many persistent ground-water pollutants by utilizing them as a source of carbon and energy (van der Meer et al. 1987; Bosma et al. 1988; Wackett and Gibson 1988; Wackett and Householder 1989; Pedit et al. 2002; Abuhamed et al. 2004; Kim et al. 2005). Thus, in situ bioremediation and biological stabilization have been promoted as promising strategies for remediation of residual contamination of ground water.

Optimum substrate metabolism by microorganisms is perhaps the most limiting factor for in situ bioremediation of contaminated ground-water aquifers (Kim et al. 2005), and requires an accurate understanding of the spatial and temporal distribution of viable microbes with respect to contaminants in the aquifer. Chemical gradients are ubiquitous in contaminated aquifers, particularly surrounding discrete pockets of low-permeability soil in which NAPLs and other contaminants remain trapped. Chemotaxis, the ability of microbes to sense and respond to chemical gradients, is one bacterial transport mechanism that can help optimize bacterial distributions in contaminated aquifers (van der Meer et al. 1987; Bosma et al. 1988; Harvey and Garabedian 1991); it can therefore be potentially exploited to enhance in situ bioremediation of contaminated aquifers (Harvey and Garabedian 1991; Singh and Olson 2008). Chemotaxis enables bacteria to move into niches that are best suited for their growth and survival (Pandey and Jain 2002) and can potentially improve in situ bioremediation by: increasing the rate of contaminant consumption by ensuring optimal conditions for metabolism; increasing the
number of metabolically active bacteria in the area surrounding the NAPL thereby further enhancing the aqueous chemical consumption rate; reducing aqueous concentrations in the surrounding bulk media, thereby increasing the chemical diffusion gradient and increasing the dissolution rate of the NAPLs; and limiting the toxic effects of aqueous chemical concentrations to bacteria by enabling them to move away from higher, toxic concentrations (Baena-Ruano et al. 2006; Singh and Olson 2008). Chemotactic bacteria can also form accumulation bands surrounding dissolving NAPLs, potentially forming a biocurtain for containing and treating dissolving plumes and improving upon designed natural attenuation studies (Singh and Olson 2008).

While viable contaminant-degrading bacteria are capable of removing contaminants via metabolism and are beneficial in contaminated aquifers, non-viable bacteria may cause bioclogging, which in turn may decrease the permeability of porous media (Lappan and Fogler 1996). This permeability reduction may cause changes in contaminant flow paths and viable bacterial transport (Cunningham et al. 1991; VandenCivere and Baveye 1992; Peyton 1996). Thus, for achieving successful bioremediation, in addition to the knowledge of bulk bacterial distributions in contaminated aquifers (Kim and Jaffe 2007), it is also important to understand the viability of these bacteria and the effects of dissolving chemicals on their survival rates.

It is well known that many ground-water contaminants that can be degraded by soil-inhabiting bacteria at low concentrations via metabolism or co-metabolism, are toxic to them above certain threshold concentrations (Collins and Daugulis 1999; Abuhamed et al. 2004; Kim et al. 2005). Prolonged exposure to higher concentrations of these contaminants may cause significant stress to bacterial cells that may result in temporary or permanent loss of their ability to degrade specific contaminants (Kim and Jaffe 2007) or even in complete loss of cell viability.
Ray and Peters (Ray and Peters 2008) reported inhibition in both anabolism and catabolism of *Pseudomonas aeruginosa* as a result of exposure to high concentrations of contaminants.

The toxic effects of volatile organic contaminants to bacteria are often measured in terms of growth inhibition (Collins and Daugulis 1999; Parales and Harwood 2002; Hamed et al. 2003). Kim and Jaffe (2005) reported growth inhibition in several *Pseudomonas* species at benzene concentrations below saturation while Wackett and Housekeeper (1989) reported TCE toxicity to *Pseudomonas putida* F1 (*P. putida* F1) where TCE was provided in the head space as vapor phase. However, in actual contaminated aquifer scenarios, contaminants remain trapped as small discrete ganglia, dispersed throughout the ground water. In close proximity to these ganglia, contaminant-degrading bacteria frequently get exposed to saturation concentrations of these contaminants. In order to predict the toxic effects of dissolving NAPL ganglia on contaminant-degrading cells, it is necessary to develop a method that can quickly and accurately test bacterial viability under prolonged exposure to saturation concentrations of ground-water contaminants.

The goal of the present study is to present a microscopic visualization of viable and non-viable *P. putida* F1 cell distributions surrounding a simulated dissolving NAPL ganglion using a live-dead plug assay experiment. Conventional chemotaxis plug assay experiments (Parales et al. 2000; Olson et al. 2004) are used to simulate dissolving NAPL ganglia in ground water. In the present study, a dual color fluorescent bacterial viability assay is used to distinguish between viable and non-viable cells. Trichloroethylene is a priority ground-water pollutant in the United States and was therefore chosen as a representative model NAPL; however the work in the present study may be extended to include other contaminant-degrading bacteria and contaminant combinations.
To evaluate the kinetics of contaminant toxicity to *P. putida* F1, a method was developed for measuring individual cell viability under contaminant stressed conditions in a zero headspace sealed environment. Changes in live and dead cell counts in samples stressed separately with TCE and toluene were quantified using fluorescence microscopy. Toxicity data for TCE and toluene were fit with linear and exponential viability-decay models to quantify the viability-decay rate of *P. putida* F1 exposed to saturated concentrations of the two contaminants. The effect of contaminant toxicity on the physiological condition of *P. putida* F1 was examined by measuring the subsequent growth of bacterial cells exposed to TCE for various time periods.

5.2. Materials and methods

5.2.1. Live-dead bacterial viability assay

Dual color fluorescent assays are widely used to investigate the viability of microorganisms (Boulos et al. 1999; Decker 2001; Mesa et al. 2003; Leuko et al. 2004; Baena-Ruano et al. 2006). The Live/Dead™ BacLight™ Bacterial Viability Kit (L7012) (Invitrogen, Carlsbad, CA, USA) used in the present study is described extensively in the literature (Boulos et al. 1999; Decker 2001; Mesa et al. 2003; Leuko et al. 2004; Baena-Ruano et al. 2006). Briefly, the kit was developed to differentiate live and dead bacterial cells based on plasma membrane permeability (Baena-Ruano et al. 2006). The kit consists of two dyes, SYTO 9 and propidium iodide (PI), both contained in a solution of anhydrous dimethylsulfoxide (DMSO) (Boulos et al. 1999). These stains differ both in their emission spectral characteristics and in their ability to penetrate healthy bacterial plasma membranes (Baena-Ruano et al. 2006). The nucleic acid dye SYTO 9 penetrates all bacterial cells and stains green, whereas PI, which is highly charged (Leuko et al. 2004), cannot penetrate intact membranes and only stains cells with compromised membranes. The presence of both dyes in a cell produces red fluorescence (Baena-Ruano et al. 2006). Thus
for the purposes of the present study, cells stained green are considered live, and those stained red are considered dead or damaged.

5.2.2. Cell culture

*Pseudomonas putida* F1, that degrades both toluene and TCE via metabolism and co-metabolism, respectively (Parales et al. 2000), was obtained from Caroline Harwood at the University of Washington. *Pseudomonas putida* F1 cells exhibit chemotaxis toward both toluene and TCE [23], and can grow on toluene, but not exclusively on TCE. For chemotaxis studies, cells from a -80 °C frozen glycerol stock were grown on Luria broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter of deionized water) in a shaking incubator. These cells were used as a seed culture to inoculate a 50 ml 1:1 (v/v) mixture of Hunter’s mineral base [28] and Luria broth in 125 ml sealed serum bottles. Prior to inoculation, sealed bottles were sparged with oxygen for 10 to 15 min and approximately 25 µl of toluene was added in the head space. For cells used in plug assay experiments, inoculated bottles were incubated at 30 °C in a shaking incubator for approximately 15 to 20 h until cells reached an optical density at 590 nm (OD\textsubscript{590}) of approximately 0.8 to 1.0 (Spectronic\textsuperscript{®} 20 GENESYS\textsuperscript{™} Spectrophotometer, Thermo Scientific; Waltham, MA, USA). Cells were washed twice with 0.85% NaCl buffer (8.5 g NaCl, 0.029 g ethylenediaminetetraacetic acid [EDTA] per liter of DI water; pH = 6.8 approximately) by centrifugation, and resuspended in 0.85% NaCl buffer to a concentration 2.5 times higher than that of the original harvest. Cells used in toxicity assays were grown on Luria broth only and likewise harvested in the middle of their exponential growth phase, washed twice and resuspended in 0.85% NaCl buffer solution. An OD\textsubscript{670} of 0.15 (\(~10^7\) cells/ml) was precisely controlled (as recommended by the manufacturer of the Live/Dead BacLight Bacterial Viability Kit), in order to achieve consistent bacterial counts in toxicity assays.
5.2.3. Cell staining protocol

The Live/Dead BacLight Viability Kit (L7012) from Invitrogen was used as a staining agent for *P. putida* F1 in the present study. A 1:1 (v/v) dye mixture of SYTO 9 and PI was added to cell samples as described above, and prepared for the live-dead chemotaxis assays and toxicity assays at ratios of 0.3% (v/v) and 0.15% (v/v), respectively. Samples were vortexed and stored in the dark for at least 15 min, with intermittent vortexing.

5.2.4. Live-dead chemotaxis plug assay

Plug assays have been widely used to determine the chemotactic response of different bacterial strains toward various environmental pollutants (Yu and Alam 1997; Parales et al. 2000; Olson et al. 2004). A plug containing 13% (v/v) TCE was mixed with 0.1 g low-melting-temperature agarose (NuSieve GTG Agarose, FMC Bioproducts, Rockland, ME, USA) in 0.5 ml 0.85% NaCl buffer in an air-tight microcentrifuge tube to avoid volatilization of TCE. The plug mixture was melted at 80 °C in a water bath. A small droplet (~ 10 µl) of melted TCE-containing agarose was placed on the top of a microscopic slide with two thin plastic strips placed on either side of the droplet, approximately 2 cm apart. A cover slip, supported by the plastic strips at the ends, was placed on top of the plug, resulting in the formation of a chamber surrounding the droplet. A suspension of stained bacteria was flooded into the chamber surrounding the plug, creating a system representative of a NAPL droplet dissolving in ground water. To investigate the tactic response of *P. putida* F1 and the effect of TCE toxicity, images were captured at one edge of the plug at different time intervals using fluorescence microscopy (Leitz Diplan, Leica Microsystems, Heidelberg, Germany) (Figure 5.1). This microscopy set-up enabled visualization of stained cells through two separate channels: green fluorescence showing live cells (Leica N2.1 filter, excitation = 515-560 nm) and red fluorescence showing dead/damaged cells (Chroma 41001 filter, excitation = 460-500 nm).
5.2.5. **Toxicity assay**

Five replicate toxicity assays were performed for both TCE and toluene to assess their toxicity to *P. putida* F1. Specifically, changes in live and dead cell counts were measured in contaminant-stressed populations of bacteria as a function of time. To evaluate the toxic effect of TCE on *P. putida* F1, two aliquots of stained bacterial suspensions were added to two clear 2 ml crimp-top borosilicate glass vials (Thermo Scientific, Waltham, MA, USA) with negligible head space. Vials with stained cells were crimp sealed with polytetrafluoroethylene rubber septa in order to avoid potential losses of TCE due to volatilization. A gas tight syringe (Hamilton Series 800 Microliter Syringe Multi-Pak, Hamilton, Reno, NV, USA) was used to inject 10 µl of TCE (~0.5% v/v) into one of the vials. This vial represented cells under TCE-stressed conditions. No TCE was added to the other vial which served as a blank sample. Approximately 10 µl of stained bacterial suspension was drawn from each vial after brief vortexing, at 30 min intervals, for the duration of the experiment. Each withdrawn sample was imaged microscopically for live and dead cells, as described in the live-dead chemotaxis plug assay protocol. Similar toxicity assays were performed using 0.5 % (v/v) toluene concentrations.

5.2.6. **Cell enumeration**

Five images were captured for enumeration of both red and green cells at five randomly selected locations for all withdrawn samples using a CCD camera (AxioCam, Carl Zeiss; Gottingen, Germany). The number of live cells (stained green) and dead/damaged cells (stained red) were counted in respective images using UTHSCSA Image Tool version 3.0 software (University of Texas Health Science Center at San Antonio, TX, USA) in the Count/Tag mode under 1:1 zoom. Cell counts for green and red cells in each sample are presented as the average of five fields of view and reported as the average number of cells per sample at that specific time.
5.2.7. Growth curves

To support the visual observation (i.e., green and red stained cells representing live and dead cells respectively), the effect of TCE toxicity on the physiological condition of *P. putida* F1 cells was investigated. Growth curve experiments were conducted to measure the ability of TCE-exposed bacteria to reproduce under favorable conditions following various exposure durations. Three vials were set in parallel, as described for the toxicity assay. A fourth vial was processed similarly, except that the re-suspension buffer was replaced with 70% isopropyl alcohol. This vial served as the dead control. Two of the first three vials, prepared above, were exposed to TCE as described for the toxicity assay and the remaining one vial served as an unstressed control and was not exposed to TCE. All the vials were briefly vortexed at half-hour intervals for consistency with the toxicity assay. After one hour, 0.5 ml aliquots were taken from the unstressed control, the dead control and one of the TCE-exposed vials, and were inoculated in Luria Broth at approximately 0.5% (v/v). A similar aliquot from the remaining TCE-exposed vial was drawn after 2.5 h and inoculated (~0.5% (v/v)) in Luria Broth. All inoculated samples were incubated at 30°C on an orbital shaking incubator (~150 rpm), for optimal cell growth. In order to avoid any growth inhibition due to the small amount of TCE that may have been present in the TCE-stressed inoculums, samples were purged with oxygen for approximately 5 min prior to incubation. To investigate the effect of staining on the physiological state of *P. putida* F1, a parallel growth curve was run, inoculated (~0.5% (v/v)) with cells taken directly from the original sample before staining. This sample was identified as the blank control as cells were neither stained nor exposed to TCE. Cell growth concentrations were monitored at OD_{590} at 1h intervals until bacteria entered the stationary growth phase. Growth curve experiments for each sample type were performed in duplicate.
5.2.8. Data analysis

The kinetics of contaminant toxicity to a microbial population is dependent on various complex interactions between microbes and contaminants, including: diffusion rates of contaminants through bacterial cell walls, microbial resistance toward contaminants, exposure history, and microbial growth conditions. In addition, Kim and Jaffe (2007) reported temporary loss of cell viability due to accumulation of contaminant material or its degradation by-products within bacterial cells. Toxicity data for live cell counts from the present study were fit into two simplified kinetic models to characterize these complex interactions in terms of simplified model parameters. The bacterial survival ratio \( S = N/N_0 \) versus time \( t \) was fitted to linear (Eq. 5.1) and exponential (Eq. 5.2) viability-decay models.

\[
S = \frac{N}{N_0} = m - kt \quad 5.1
\]

\[
S = \frac{N}{N_0} = \exp(-kt^n) \quad 5.2
\]

where, \( N \) is the live bacterial count at any time, \( t \); \( N_0 \) is the initial live bacterial count; \( k \) is viability decay constant; and \( m \) and \( n \) are empirical constants.

Non-linear least squares regression, in which the sum of the squares of the differences between observed and predicted survival ratios was minimized, was used to find optimal values of the kinetic parameters \( k \) and \( n \).

5.3. Results

5.3.1. Chemotaxis and viability of \textit{P. putida} F1 in a TCE gradient

Figure 5.1 illustrates the TCE-triggered chemotactic response of \textit{P. putida} F1 toward TCE (row 1), as well as the toxic effect of TCE on \textit{P. putida} F1 (row 3). In order to observe the bacterial response to diffusing TCE, images of live cells of \textit{P. putida} F1 were taken at 1, 5, 10, and 20 min after flooding the bacterial suspension into the plug assay chamber (Figure 5.1, row 1). Image J
was used to plot normalized (with respect to initial average intensity values, averaged over the field of view, farther away from the plug in the image taken at $t = 1$ min) intensity values, averaged over 100 pixels, of the live bacterial concentration profiles for each image captured (Figure 5.1, row 2). All the fluorescent images were converted into grey scale images before plotting intensity profiles. To obtain green fluorescence intensity profiles, red fluorescence images were subtracted from corresponding green fluorescence images and vice-versa. Initially, 1 min following bacterial introduction into the chamber, a nearly uniform profile of live cells can be observed surrounding the plug. A band of live bacterial accumulation, at a short distance away from the edge of the plug, can be noticed after 5 min, which is observed to migrate away from the TCE-containing plug over time (10 and 20 min). Images taken at identical time periods with the red filter indicate dead or damaged cells. Red fluorescence intensity and hence dead/damaged cell concentration in the respective images increases over time directly adjacent to the TCE-containing plug (Figure 5.1, row 3). Changes in normalized red intensity profiles (similar to those for green fluorescence images) of the corresponding images can be observed in Figure 5.1, row 4, suggesting an accumulation of dead cells.

Similar experiments were performed for control plug assay experiments, where no TCE was added to the agarose plug. Images were captured for both live and dead cells at identical time periods as in the TCE-containing plug assay experiments. Live and dead cell intensity profiles remained primarily uniform over the course of the experiment, and did not follow patterns observed in the TCE-containing plug assays (see the supplemental data, Figure B.1 in Appendix-B, for detailed information).

5.3.2. **Live-dead toxicity assay**

Figure 5.2 represents images of stained *P. putida* F1 cells during the course of a typical toxicity assay at different time intervals. When TCE was introduced into the sample, the concentration
### Table 5.1: Chemotactic Response and TCE Toxic Effect

<table>
<thead>
<tr>
<th>Time</th>
<th>t = 1 min</th>
<th>t = 5 min</th>
<th>t = 10 min</th>
<th>t = 20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemotactic response (live cell accumulation bands)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Normalized intensity profiles of live cells</td>
<td><img src="graph1.png" alt="Graph" /></td>
<td><img src="graph2.png" alt="Graph" /></td>
<td><img src="graph3.png" alt="Graph" /></td>
<td><img src="graph4.png" alt="Graph" /></td>
</tr>
<tr>
<td>TCE toxic effect (dead cell accumulation bands)</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>Normalized intensity profiles of dead cells</td>
<td><img src="graph5.png" alt="Graph" /></td>
<td><img src="graph6.png" alt="Graph" /></td>
<td><img src="graph7.png" alt="Graph" /></td>
<td><img src="graph8.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

**Figure 5.1:** Live-dead bacterial plug assay experiment showing the chemotactic response of *Pseudomonas putida* F1 (*P. putida* F1) to trichloroethylene (TCE) diffusing from an agarose plug (row 1) and the toxic effect of high TCE concentrations on *P. putida* F1 (row 3) at 1, 5, 10, and 20 min. after bacterial introduction into the chamber. Dark semicircular regions on the left side of each image (rows 1 and 3) represent plugs that contain 13% (v/v) TCE, that diffuses out into the surrounding aqueous suspensions of stained (live, in row 1, and dead/damaged, in row 3) bacteria. Rows 2 and 4 illustrate normalized live bacterial concentration- and dead/damaged cell concentration- profiles of images in Rows 1 and 3, respectively; where the horizontal axis in each panel represents distance from the plug edge (number of pixels).
of live cells was observed to decrease (Figure 5.2a-c), accompanied by a corresponding increase in dead/damaged cell concentrations (Figure 5.2d-f). Initially there were almost no visible dead cells present in the sample (Figure 5.2d) and after 2.5 h (i.e., at the end of the experiment) almost all of the live cells had disappeared (Figure 5.2c). Bacterial survival ratios calculated based on microscopic analyses of the live-dead bacterial viability assay were verified using flow-cytometry analysis (FACSARia cell sorter; BD Biosciences, San Jose, CA, USA) and results obtained correlated well.

In parallel unstressed control samples (i.e., no TCE was added to stained cells), there were no significant changes in the concentration of green cells over time, and no red cells were observed during the course of the toxicity assay experiment (images not shown).

5.3.3. Toxicity kinetics

Survival data for *P. putida* F1 in TCE-stressed samples and unstressed blanks are plotted in Figure 5.3a. In TCE-stressed samples, a seven-fold decrease in the survival ratio (from 1 to 0.14) was observed after 2.5 h of exposure. However, no definite trend was observed in corresponding blank samples, where the normalized survival ratio remained constant at approximately 1, for the same exposure time (Figure 5.3a). Thus any effects of the staining agents (SYTO 9 and PI) on bacterial viability may be neglected. Trichloroethylene toxicity data were fitted to two candidate models (Eq. 5.1 and Eq. 5.2) and the best fitted model parameters, along with $r^2$ values are presented in Table 5.1 and plotted in Figure 5.3a. The coefficient of determination for the best fit exponential model ($r^2 = 0.965$) was significantly higher than for the linear model ($r^2 = 0.730$), predicting a viability-decay constant of $k_{TCE} = 0.025 \text{ h}^{-1.95}$.

The effect of toluene exposure on bacterial survival for different exposure durations is shown in Figure 5.3b. In comparison with TCE, the bacterial survival ratio decreased more slowly
Figure 5.2: Green- (representing live cells at (a) t=0, (b) t=1h and (c) t=2.5h) and red- (representing dead cells at (d) t=0h, (e) t=1h and (f) t=2.5h) stained *Pseudomonas putida* F1 cells during a typical trichloroethylene toxicity assay.

Table 5.1: Best fit linear and exponential (Eq. 1 and 2, respectively) model parameters for *Pseudomonas putida* F1 survival data following trichloroethylene and toluene exposure, with corresponding $r^2$ values. $k$ is a toxicity constant and $m$ and $n$ represent empirical constants.

<table>
<thead>
<tr>
<th>Contaminants</th>
<th>Model</th>
<th>Parameters</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$m$</td>
<td>$k$</td>
</tr>
<tr>
<td>Trichloroethylene</td>
<td>linear</td>
<td>1.0</td>
<td>0.262</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exponential</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>0.198</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exponential</td>
<td>-</td>
</tr>
</tbody>
</table>
for toluene exposure. Linear (Eq. 5.1) and exponential (Eq. 5.2) best-fit models for toluene toxicity to *P. putida* F1 are shown in Figure 5.3b, and corresponding best-fit modeling parameters are presented in Table 5.1. In this case, the linear model ($r^2 = 0.973$) was marginally better fit to the survival data than the exponential model ($r^2 = 0.967$) with viability-decay constant $k_{\text{toluene}} = 0.198 \text{ h}^{-1}$ and was therefore chosen as a representative model of toluene toxicity.

5.3.4. Cell viability

The effect of TCE exposure on subsequent growth of *P. putida* F1 under favorable conditions was assessed by comparing growth curves of stained cells, cells exposed to TCE for two different durations, an unstressed control (i.e., stained cells not exposed to TCE) and a dead control (i.e., stained cells suspended in isopropyl alcohol for one hour, as recommended by the manufacturer of the Live/Dead BacLight Bacterial Viability Kit) (Figure 5.4). To investigate the effect of staining, blank controls and unstressed control samples were compared. Growth curve experiments showed similar patterns of cell growth for all samples tested and are presented in Figure 5.4. Unstressed controls lagged approximately 1h, as compared with blank controls, however TCE exposure caused a further increase in the lag time which was more pronounced with longer TCE exposure. No growth was observed in the dead control during the course of the growth curve experiments (Figure 5.4). Samples exposed to TCE for 1h and 2.5h displayed lag times in growth ranging from 2 to 4h, with respect to blank control experiments. However, it can be observed that the stationary concentrations were similar for all cases.

5.1. Discussion

Many soil-inhabiting bacteria, such as *P. putida* F1, exhibit chemotaxis toward and have the ability to degrade TCE and toluene (Yu and Alam 1997; Parales et al. 2000; Olson et al. 2004). As discussed earlier, exposure of these bacteria to high concentrations of contaminants is common
Figure 5.3: Survival ratios ($S = N/N_0$) of *Pseudomonas putida* F1 in trichloroethylene- (a) and toluene- (b) stressed samples (observed (◊), linear model (—) and exponential model (---)) and non-stressed samples ( ) at different time intervals throughout toxicity experiments. Error bars represent standard errors for five replicate experiments.
Figure 5.4: Effect of staining and different trichloroethylene (TCE) exposure durations on growth curves of *Pseudomonas putida* F1 measured in terms of OD$_{590}$. Blank control (◊) culture was neither stained nor exposed to TCE, ‘unstressed control’ (□) was stained but not exposed to TCE, dead control (●) was resuspended in 70% isopropyl alcohol and remaining cultures were exposed to TCE for 1 hr (○) and 2.5 hrs (△).
in contaminated aquifers, particularly surrounding dissolving NAPL ganglia. The live-dead plug assay experiments introduced in the present study simulates such a scenario. It can be observed in Figure 5.1 that 5 min after bacterial introduction into the chamber, the intensity of green fluorescence (representing live cells) appears to increase a short distance away from the plug, compared with the baseline uniform intensity seen at 1 min. This observation can be attributed to a tactic bacterial response to the TCE gradient formed surrounding the plug, as no such accumulation was observed in experiments with blank plugs (Figure B.1, Appendix-B). This characteristic band of bacterial accumulation has been observed in previous plug assay experiments, always a short distance away from the actual plug (Parales et al. 2000; Olson et al. 2004). However, the fate of the bacteria immediately adjacent to the plug was not known in these studies. A portion of the live bacteria adjacent to the plug may have migrated away from the plug to avoid high aqueous concentrations of TCE while those further away from the plug may have moved toward the plug in response to the increasing chemical gradient. Over time, as more TCE diffused from the plug into the chamber and the high TCE concentration front propagated farther away from the plug into the bacterial chamber, the corresponding green fluorescent intensity band migrated further away from the plug (10 min after bacterial introduction) and eventually disappeared (20 min). Changes in both green and red color intensities from 10 to 20 min were more pronounced than those from 1 to 10 min. This observation may be attributed to a delay in TCE diffusion from one medium to another (i.e., from the plug to the aqueous bacterial suspension).

These observations suggest that live bacteria either moved out of the observed field of view or died as a result of the toxic effect of TCE. The increase in red fluorescence (representing dead cells) immediately adjacent to the TCE plug suggests loss of cell viability (Figure 5.1). A very low intensity of red fluorescence after 1 min and 5 min suggests few dead cells initially
surrounding the plug. As more and more TCE diffuses into the surrounding aqueous medium (10 min and 20 min after bacterial introduction), an increase in red fluorescence intensity can be observed. In experiments conducted with blank plugs, no change in red fluorescence intensity was observed. These observations support the assertion that exposure to high concentrations of TCE for prolonged time can cause toxic effects to *P. putida* F1 cells.

The kinetics of contaminant toxicity are important in understanding the impact of ground-water pollutants on indigenous bacteria. In toxicity studies, *P. putida* F1 showed an initial resistance to TCE, as the survival ratio (i.e., the ratio of live cells to the original live population) did not decrease significantly for 1 to 1.5 h of TCE exposure (Figure 5.3a). Such an effect can be attributed to mass transfer limitations in our assays, or in a delay in the diffusion of TCE into bacterial cells. Similar lags or delays of disinfectants into microbial cells have been discussed in the literature during water treatment processes (Gyurek and Finch 1998). Such a toxicity lag was not evident due to toluene exposure (Figure 5.3b). Different toxicity patterns of TCE and toluene to *P. putida* F1 may be attributed to the smaller size of toluene molecules in comparison with TCE molecules that might have resulted in slower penetration of TCE molecules into bacterial cells. However, once an initial loss in viability is observed, TCE seems to affect bacterial viability more quickly than toluene. As seen in Figures 5.3a and 5.3b, approximately one log reduction in the overall bacterial survival ratio was observed over 2.5 h in TCE-stressed samples in comparison with 4.0 h for toluene-stressed samples. It is possible that differences in the partitioning coefficients ($K_{ow}$) for TCE and toluene affect uptake rates and toxicity kinetics. Investigation of such effects is beyond the scope of this work, but is recommended for better understanding of toxicity mechanisms.

To test the validity of the best fit models, both TCE and toluene toxicity experiments were repeated with 1.0 % (v/v) concentrations. The survival data obtained are plotted in Figure
5.5 along with the respective model predicted values, for best-fit model parameters (Table 5.1), for similar exposure times. The best-fit linear model (Eq. 5.1) captured toluene toxicity to *P. putida* F1 well, which is evident from coinciding observed and model-predicted values (Figure 5.5). However, the best-fit exponential model (Eq. 5.2) for TCE slightly over predicted the toxic effect of TCE as compared with experimental observations (Figure 5.5). This effect may be due to the larger volume of TCE used in these experiments, which may have increased the overall toxic effect prediction by increasing the number of *P. putida* F1 cells that came in direct contact with TCE droplets.

Finally, the effects of TCE exposure for different durations on subsequent cell growth were examined. Inhibition in the growth of *P. putida* F1 cells due to TCE exposure was observed, and was more pronounced for longer exposure times. This delay in growth due to TCE exposure may be attributed to one or more of the following explanations. First, samples exposed to TCE for longer durations had fewer live cells in the inoculum and hence took longer to reach the exponential growth phase (i.e., longer lag phase for longer TCE exposure). Additionally, the delay in growth of *P. putida* F1 may be due to a temporary change in the physiological condition of the live cells due to TCE exposure, from which they recovered when suitable growth conditions were provided. Plate count experiments confirmed similar delays in colony formation in TCE-stressed samples as compared with non-stressed samples. Recovery of injured cells under suitable growth conditions has been reported previously (Kim and Jaffe 2007). This temporary loss of cell viability may be due to excess accumulation of TCE inside the cells; cells may re-grow after an initial lag period that allows them to consume the accumulated contaminant, which is in agreement with the observation made by Kim and Jaffé (2007). Cells exposed to isopropyl alcohol did not recover from injury and no cell growth was observed during the 24-h incubation period. Cultures in the present study were able to repopulate when TCE-
stressed cells were grown under suitable growth conditions and in the absence of stressor. This response contrasts with previous toxicity studies, in which stressors were present during growth curve experiments, possibly requiring significant energy for cellular repair, and stressed samples leveled off at lower concentrations than unstressed samples (Wackett and Householder 1989; Hamed et al. 2003; Kim et al. 2005).

The present study provides a microscopic evaluation of TCE and toluene toxicity to P. putida F1. Compared with traditional methods using growth inhibition as a proxy for toxicity, the present study directly correlates contaminant exposure with cell viability and validates a predictive model of bacterial survival. Toxicity reports based on growth rate inhibition may represent temporary loss of reproducibility or limited cell division due to redistribution of bacterial resources in stressed conditions, formation of reactive intermediates leading to modification of intracellular molecules (Wackett and Householder 1989), and accumulation of substrate and/or intermediate oxidation products above inhibitory levels (Tempest and Neijssel 1978). Any of these stressors may inhibit growth, which can be subsequently regained upon exposure to nonselective media (Kim and Jaffe 2007), but may not necessarily result in cell death. The toxicity assay presented in the present study is based on cell membrane integrity and may be more useful in accurate toxicity predictions. Toxicity models presented in the present study may be used to design improved bioremediation strategies by optimizing the exposure time to contaminant-degrading cells. Issues relating to long-term bacterial viability and reproduction ability, and the effect of toxicity with respect to ultimate contaminant biodegradation potential, need to be studied in further detail.
Figure 5.5: Observed survival ratio ($S = N/N_0$) of *Pseudomonas putida* F1 with 1% (v/v) trichloroethylene (TCE)- (×) and toluene- (◊) stressed samples. (---) and (—) represent best fit model prediction values for TCE (exponential) and toluene (linear), respectively. Error bars represent standard error in the survival ratio for triplicate experiments.
CHAPTER-6: FIELD-SCALE APPLICATION OF CHEMOTAXIS TO GROUNDWATER BIOREMEDIATION

6 Background

Bacterial movement in soil as a result of chemotaxis and random motility is well documented by soil microbiologists (Soby and Bergman 1983; Bashan and Holguin 1994). However, studies demonstrating direct evidence of enhanced bioremediation at the field scale due to chemotaxis are very rare. Witt et al. (Witt et al. 1999) demonstrated faster migration of Pseudomonas stutzeri KC, a denitrifying strain chemotactic toward nitrate, in comparison to groundwater flow velocities in a bench scale study of carbon tetrachloride (CT) degradation. CT and nitrate were injected with groundwater into a model aquifer column containing CT-saturated sediments. Bacteria and tracers were inoculated at the top of a column and it was observed that bacteria migrated through the column faster than the traces, removing both adsorbed and aqueous CT. This enhanced migration of strain KC was attributed to the chemotactic response as a result of nitrate depletion in the vicinity. Olson et al. (Olson et al. 2004) also reported chemotaxis of P. putida F1 toward TCE in small scale laboratory columns packed with glass-coated polystyrene. Two field scale bacterial transport studies recently reported faster transport and greater recovery of motile Pseudomonads compared with non-motile bacterial strains (Becker et al. 2003; Ford and Harvey 2007). However, no distinction between chemotaxis and motility was made in these two field studies. Paul et al. (Paul et al. 2006) evaluated chemotaxis of Ralstonia sp SJ98 toward p-nitrophenol in soil microcosms using qualitative and quantitative plate and tray assays. However, no effort was made to evaluate the effect of chemotaxis on biodegradation. Recently, enhanced bacterial migration in bench and field scale model aquifers have been demonstrated (Wang et al. 2008; Strobel et al. 2011). The coupled effect of
chemotaxis and growth on microbial distribution in organic amended aquifer sediments was studies by (Wang et al. 2008). They have compared their bench and field scale model aquifers data and concluded that the combined effect of both chemotactic bacterial motility and growth greatly accelerate the migration of bacteria due to chemoattractant gradient in vertical direction. (Strobel et al. 2011) have reported greater vertical migration and apparent transverse dispersion of chemotactic bacteria in comparison to non-chemotactic mutant of same bacterial species in their bench scale sand filled aquifer model.

One concern for in situ bioremediation is the bioavailability of contaminants, or the proximity of target pollutants to degrading microorganisms. Many subsurface pollutants are hydrophobic, sparingly soluble in groundwater, and form a non-aqueous-phase liquid (NAPL) (Bedient et al. 1997). These NAPLs remain trapped in low permeability regions in heterogeneous subsurface environments, making them difficult to remove by conventional pump-and-treat treatment methods. Chemotaxis is a mechanism for bringing cells in close proximity to contaminants (Samanta et al. 2000; Parales and Harwood 2002; Ford and Harvey 2007) thereby reducing limitations in bioavailability due to mass transfer limitations or low contaminant solubility (Parales and Harwood 2002). It also enables bacteria to adjust their proximity to toxic repellents, thereby increasing their odds for survival and optimizing their distribution in conditions favorable for bioremediation. A vigorous chemotactic response can enhance the availability of carbon and/or energy resources significantly and hence chemotactic bacteria can grow faster than their non-chemotactic counterparts. Faster chemoattractant consumption causes localized depletion of the contaminant which creates even steeper chemical gradients that trigger higher driving forces for dissolution of the contaminant. In light of this, groundwater treatment technologies can take advantage of bacterial chemotaxis for enhancement of
contaminant removal. We propose some scenarios of groundwater remediation in which chemotaxis can potentially be exploited to enhance biodegradation are described below.

6.1 Enhanced remediation due to chemotaxis in heterogeneous porous media

Contaminants often remain trapped in pockets of low permeability within the subsurface. Figure 6.1 depicts a contaminated aquifer scenario where contaminant is trapped in a low permeability clay lens. In Figure 6.1(a), non-chemotactic degradative bacteria flow with advective ground water through the surrounding high permeability region, limiting remediation to the slow diffusion of contaminants into the high permeability region. Lanning et al. (Lanning et al. 2008) have reported that in bulk liquid chemotactic bacteria can swim transverse to the flow direction at fluid velocities greater than typical ground-water flow velocities. Long and Ford (2009) further verified transverse chemotactic migration of bacteria in porous microfluidic ‘T-sensor’. We have also observed similar transverse chemotactic migration and accumulation of bacteria in low permeability region of a dual permeability porous microfluidic device during this study (Chapter 4). Thus chemotactic bacteria flowing with ground water in the high permeability regions can sense chemical gradients induced by diffusing contaminants and migrate toward the source (Figure 6.1(b)). Chemotactic bacteria are able to swim upgradient in typical ground-water velocities (Long and Hilpert 2007) to penetrate low permeability regions from the bulk flow in high permeability areas (Olson et al. 2006). Accumulation of bacterial bands surrounding contaminated low permeability regions can significantly enhance contaminant removal.

6.2 Application of chemotaxis to bioaugmentation

Introduction of beneficial microorganisms into contaminated aquifers for the purpose of enhancing biodegradation is referred to as bioaugmentation. Bioaugmentation is highly site-specific and dependent on the microbial ecology and physiology of the site (USEPA 1998),
Figure 6.1: Contaminant removal from low permeability region of a heterogeneous porous medium. Non-chemotactic contaminant degrading bacteria flowing with groundwater remain dispersed across the aquifer (A) while chemotactic bacteria respond to the chemical gradient formed as a result of contaminant diffusion out of the low permeability region and accumulate around the low permeability region (B). This bacterial accumulation around the low permeability region may enhance the contaminant consumption rate which can result in even steeper gradient and faster dissolution of contaminant as a result.
however it is feasible for combined chemotaxis and genetically improved degradation capabilities to significantly improve the remediation rate of an aquifer.

Figure 6.2 shows a schematic of a bioaugmentation strategy that takes advantage of chemotaxis for improved contaminant removal from an aquifer contaminated due to leakage from an underground storage tank. Ground-water flow past the contaminated region will slowly dissolve the contaminant and a contaminant plume will form that may contaminate the entire aquifer system. Figure 6.2(a) represents the initial step of a bioaugmentation scheme. Injected non-chemotactic bacterial strains would consume the contaminant plume in the vicinity of an injection point or would rely on contaminant advection away from the injection point for further consumption (Figure 6.2(b)). Formation and movement of chemotactic bacterial bands in a uniformly distributed contaminated region (such as a contaminant plume) has been demonstrated (Long and Hilpert 2007). A moving bacterial band can migrate upgradient and/or downgradient against contaminant gradients that are moving with ground water to overcome mass transfer limitations (Figure 6.2(c)). Moving bacterial bands capitalize on naturally available nutrient resources (such as electron acceptors) within the aquifer while non-chemotactic strains depend solely on the addition of external nutrients to stimulate the biodegradation process. Since chemotactic bacteria have the ability to swim against typical ground-water flow velocities (Long and Hilpert 2007; Lanning et al. 2008), a given bacterial washout rate may be slower for chemotactic strains compared with non-chemotactic strains, which would ultimately result in less frequent bacterial and nutrient injections. Bioaugmentation using chemotactic bacteria may significantly accelerate the degradation rate in contaminated aquifers and reduce overall remediation costs. Appropriate bacterial transport models, including chemotaxis transport parameters, will be helpful in selecting appropriate injection locations and rates to fully exploit the benefits of chemotaxis.


**Figure 6.2:** Chemotaxis assisted bioaugmentation (injecting microbial cultures into aquifer) and/or biostimulation (adding nutrients to stimulate growth of existing subsurface microorganisms) strategy for an aquifer contaminated with leaking underground storage tank. Panel on the left (A) represents initial bacterial/nutrient addition to a contaminated site. Non-chemotactic bacteria remain dispersed in the contaminated reason and may wash away with flowing groundwater (B). However, chemotactic bacteria respond to the gradients created as a result of contaminant consumption by them, and their ability to swim against and transverse to flow direction allow them to move toward higher concentration of contaminant in both longitudinal and transverse directions (C).
6.3 Chemotaxis in monitored natural attenuation (MNA)

Natural attenuation of contaminants relies on various naturally occurring in situ physicochemical and biological processes. A careful evaluation of these processes to achieve site-specific remediation objectives within a reasonable timeframe is termed monitored natural attenuation (MNA). These in situ processes, under favorable conditions and without human intervention, may cause stabilization, transformation or complete destruction of contaminants (USEPA 2000). Chemotaxis may helpful in enhancing these natural processes by facilitating bacteria to move in the regions that are best suited for their degradation capabilities and thereby faster contaminant removal rate. Therefore, chemotactic migration may be useful in overcoming mass transfer and nutrient limitations in biodegradation process by forming moving bacterial bands and incorporating such bacterial migration processes in MNA prediction models may significantly reduce the remediation time of contaminated sites.

6.4 Application of chemotaxis for contaminant containment - a variable length biocurtian

Preventing off-site migration of contaminants with ground-water flow thereby containing the source for biodegradation is known as contaminant containment. In practice, barriers such as slurry walls and reactive barriers in conjunction with continuous or intermittent pumping are used for containment of contaminated sites. A biocurtain is a biologically active zone around a contaminated region designed to contain and remediate the site. A biocurtain containing chemotactic bacteria can be advantageous by increasing the length of the reactive zone. Changes in ground-water flow conditions may occur due to pumping failures or extreme precipitation events. Chemotaxis enables bacteria to adjust their position with respect to changes in the physical and chemical characteristics of the contaminant plume, thereby reducing the risk of off-site migration. In addition, since chemotactic bacteria can sustain higher flow velocities (Long and Hilpert 2007; Lanning et al. 2008) pumping costs associated with contaminant containment can be reduced by decreasing pumping rates and frequency.
CHAPTER 7: SUMMARY AND CONCLUDING REMARKS

7.1 Summary and conclusions

Bioremediation is often limited by inadequate distribution of bacteria in contaminated regions, leading to unfavorable conditions for pollutant degradation. To design successful bioremediation schemes for contaminated regions, it is important to ensure appropriate interaction between contaminants and bacteria under suitable environmental conditions. This can be achieved by promoting processes that either facilitate spreading of contaminants so that they can be accessible to a larger number of bacteria, or enable bacteria to move into the regions of optimal contaminant concentrations.

The research efforts in this dissertation focus on investigating if bacterial motility mechanisms, random motility and chemotaxis, can assist in redistribution of both contaminant and bacteria so that the rate of biodegradation can be enhanced. We chose a microfluidic experimental setup, because of its ability to provide microscopic visualization at the pore scale along with precise and accurate measurements of transport parameters for these investigations. Three main research questions were investigated: (1) does bacterial random motility in the aqueous medium enhance contaminant mixing in porous media, (2) does chemotaxis help bacteria move toward a contaminant source diffusing from a low permeability region of a dual-permeability porous medium, and (3) does contaminant toxicity affect the distribution and viability of chemotactic bacteria? The findings relevant to each of these research questions are discussed in detail in Chapter 3, Chapter 4, and Chapter 5, respectively. Some of the key findings are summarized in the following points under four different sub headings:
7.1.1 Simulation of groundwater contamination scenario in microfluidic devices

1. A novel design for a bi-layer porous microfluidic device that simulates an environmentally relevant scenario of groundwater contamination due to leaking underground storage tank (UST) was created. The fabrication of this device was successfully achieved in our lab and the microfluidic setup was able to simulate a 2-dimensional contaminant plume formation in the simulated aquifer.

7.1.2 Contaminant mixing enhancement

2. An enhancement in contaminant (fluorescein isothiocyanate (FITC) labeled dextran as surrogate) mixing due to bacterial (*Escherichia coli* (E. coli) HCB33) random motility was observed in porous microfluidic devices. In uniform grain size with large pore throat device (MFD-I), motile bacteria contributed to a significant (2.3 fold) increase in measured apparent transverse dispersivity ($\alpha_{\text{app}}$) as compared to experiments with no bacteria, whereas non-motile bacteria only marginally (1.3 times) increased the apparent transverse dispersivity ($\alpha_{\text{app}}$).

3. Due to bacterial random motility, a 3-fold increase in the apparent transverse dispersivity ($\alpha_{\text{app}}$) was observed in a non uniform grain size with restricted pore space device (MFD-II) with restricted pore bodies and low porosity while no appreciable change was observed in a uniform grain size with small pore throat device (MFD-III) with decreased porosity and pore throat spacing.

4. Both pore throat size and pore arrangements are critical factors for contaminant mixing in porous media.

5. Dispersivity is generally considered an intrinsic property of the porous medium. Results from this study show three different values of apparent transverse dispersivity for similar porous media under three different experimental conditions (with bacteria, with non-motile
bacteria, and with no bacteria) when the same dispersant (FITC-dextran) was used. Therefore, we suggest that dispersivity may be considered as a system-specific parameter, incorporating effects of both the porous medium and dispersant, and characteristics of the transporting fluid. Therefore the term ‘apparent dispersivity’ may be used to represent dispersivity of the specific system.

7.1.3 Bacterial chemotaxis

6. Bacterial (Escherichia coli (E. coli) HCB33) chemotactic migration (in response to the chemoattractant DL-aspartic acid) toward a low permeability region was observed in the dual-permeability microfluidic device (MFD-IV). Chemotaxis significantly increased (1.09 to 1.74 times) the observed bacterial counts in low permeability region of the MFD-IV with a chemoattractant source, as compared to experiments with no chemoattractant.

7. A large increase in total bacterial counts in the pore throats just outside the low permeability region was also observed in experiments under chemotactic conditions which coincides with the predicted tails of chemo-attractant (DL-aspartic acid) concentration profiles. In contract relatively small peaks of total bacterial counts were observed in experiments under non-chemotactic conditions right at the interface of the two permeability regions which can be attributed to limitations to bacterial random motility in low permeability media.

8. The observed chemotactic response decreased linearly with increasing flow velocity. The observed chemotactic response was offset by the advective flow at the highest velocity (Darcy velocity = 0.22 mm/s) tested.

7.1.4 Toxicity kinetics of contaminant

9. The distribution of viable bacteria (Pseudomonas putida F1 (P. putida F1)) in chemical gradients and the toxic effects of prolonged exposure to high concentrations of model
contaminants (trichloroethylene (TCE) and toluene) were evaluated. Bacteria immediately adjacent to a TCE source became non-viable while cells further away used chemotaxis to accumulate in bands and eventually migrate away from the source to more favorable conditions.

10. Toxicity of TCE to *P. putida* F1 was best described with an exponential viability-decay model, with a viability-decay constant $k_{TCE} = 0.025 \text{ h}^{-4.95}$ ($r^2 = 0.965$). Toluene toxicity showed a marginally better fit to the linear viability-decay model ($r^2 = 0.972$), with a viability-decay constant $k_{toluene} = 0.198 \text{ h}^{-1}$.

11. Best-fit model parameters obtained for both TCE and toluene were used to predict bacterial viability in toxicity experiments with higher contaminant concentrations and matched well with experimental data.

### 7.2 Significance of the work:

The findings of this research have the potential to significantly improve upon several common limitations to *in situ* bioremediation, if appropriately optimized and incorporated into remediation plans for specific sites. Effective exploitation of both random motility and chemotaxis may be helpful in improving current treatment technologies such as bioaugmentation/biostimulation, monitored natural attenuation and contaminant containment to improve overall remediation efficiency, as discussed in chapter 6.

Results of the ‘contaminant mixing’ study may help better explain mass transfer mechanisms in porous media. Most contaminant transport studies at the laboratory and field scale involve a separate tracer test as a surrogate for contaminants and do not consider the effects bacteria in the system during tracer tests. Predicted contaminant transport based on these studies will be erroneous as they do not include mixing due to bacterial motility. Thus, results from this study may stimulate an interesting area of research that has not yet been
explored to the best of our knowledge. Also, it was observed that the pore arrangement and therefore soil type, texture etc. are critical factors to be considered while incorporating mixing enhancement.

Low permeability regions, such as aquitards, are difficult to treat as traces of contaminants remain trapped in them and may form chemical gradients as contaminants slowly diffuse out into surrounding groundwater. The results of the ‘bacterial chemotaxis’ study have shown that bacteria can penetrate into low permeability regions and may be helpful in remediation of the pollutant sources in these regions. Results also show that the interfaces of two permeability zones may represent critical locations to attack where large bacterial accumulation was observed. This increased bacterial concentration may enable faster contaminant dispersion rates into low permeability regions via faster degradation as well as additional mixing due to bacterial motility.

Contaminant toxicity studies may be used to predict bacterial accumulation and viability near NAPL sources, and may be helpful in designing bioremediation strategies for sites contaminated with residual NAPLs. Toxicity models presented in this study may be helpful in optimizing the exposure time to contaminant-degrading cells and therefore ensuring bacterial viability, which is equally as important as bacterial transport.

Overall, the research topics presented in this dissertation have given insight into some of the most critical factors such as mass transfer enhancement, contaminant availability, and toxicity of contaminants to degrading bacteria that often limit the success of the bioremediation process. Also, results from this study may help environmental engineering professionals evaluate the potential success of bioremediation schemes; contaminated sites seeded with motile and chemotactic bacteria are more likely to be effectively remediated due to the
enhanced contaminant bioavailability enabled by motile bacteria. Figure 7.1 shows the advantages of how bacterial motility can be exploited for enhancement of bioremediation.

7.3 Future research recommendations/Beyond this research

This research has highlighted some the areas which may be critical in improving bioremediation technology. However, there are several unanswered questions and challenges that warrant further study and may help make this research more applicable at the field scale. Some of the immediate research questions that need to be answered are discussed below:

One of the major shortcomings in most groundwater studies so far, including this one, is that tracer dyes are used as a surrogate for contaminant transport predictions. Colored tracer dyes are used instead of colorless contaminants that cannot be dyed in aqueous solution. The implicit assumption in these studies is that the tracer transport behavior is similar to that of the contaminant of interest. This assumption may not be true because of the fact that the tracers used in most of the studies are conservative and therefore largely unreactive with the transport media. However, conservative contaminants/chemicals are rare in nature and may have entirely different responses to the transporting media characteristics, such as hydrophilicity/hydrophobicity, surface roughness, and even porosity and pore-geometry (related to capillary action), in comparison to the conservative tracer. These differences may result in erroneous prediction of the contaminant transport. This effect may become even more pronounced while studying micro-scale transport processes such as chemotaxis that depend on localized chemical gradients at the pore scale (Schnaar and Brusseau 2005).

With the advent of microfluidics, where the fabrication materials generally used are glass and PDMS, surfaces can be easily oxidized to produce Si-(OH) groups (Weibel and Whitesides 2006) and therefore may be readily available as binding sites for a variety of chemicals. Additionally, advanced physical and chemical deposition techniques, such thermal deposition,
Figure 7.1: Schematic showing limitations to bioremediation that can be potentially enhanced due to bacterial motilities and their overall implication in improving bioremediation processes.
sputtering, low pressure- and plasma enhanced- chemical vapor deposition, allow great control of the surface modification of microfluidic devices. With these advantages, microfluidic devices may have the potential to be used as biosensors in biomedical, clinical, and environmental monitoring (Madou 2002). One convenient way to facilitate chemical monitoring in environmental engineering and groundwater remediation applications may be fixation of the surfaces of microfluidic devices with suitable receptors that can detect the chemical species of interest. Some of the basic requirements for such a sensing device are: the receptor layer deposited on the surface should be thin, uniform, compact, stable, and robust; it should be covalently anchored on the surface at one end; and it should have enough freedom to be able to react freely with the environmental species of interest at the other end (Raiteri et al. 2001).

In light of these advantages we have experimented with developing a novel microfluidic system that would allow visualization of both bacteria and contaminants simultaneously. Such a device would be helpful in precisely correlating bacterial behavior at the microscale with contaminant/chemoattractant concentrations, as is important when quantifying chemotaxis. The objective of this work was to immobilize concentration-sensitive dyes/chemicals onto the surfaces of microfluidic devices that would allow for visualization of changes in the dissolved concentrations of contaminants/attractants flowing through the device. This capability of the device would facilitate simultaneous imaging of chemical/chemoattractant gradients along with bacterial distributions under chemotactic conditions in the system. Results from the proposed novel visualization system could be used to further validate Darcy scale chemotaxis mathematical models.

Preliminary experiments have been performed to coat surfaces of microfluidic channels as well as PDMS and glass surfaces individually with chemoattractant sensitive dyes/chemicals. Detailed procedures, observations, and recommendations are presented in Appendix-C.
We have experimentally demonstrated chemotactic bacterial migration in microfluidic devices in an idealized lab environment. However, in order to be able to make use of these results for field scale applications it is important to develop numerical models to simulate the experimental data obtained in this study so that parameters for field scale predictions can be obtained. Finite difference numerical modeling approaches were used by Long and Ford (2009) for simulation of results obtained from microfluidic transport studies. The model was not able to capture the experimentally obtained chemotactic response of bacteria, which the authors attributed to the pore dynamics and shear stress in the system. Ahmed et al. (2010) further argued that such flow-system devices result in unsteady chemical gradients and bacteria may encounter continuous changes in gradient as they travel down the channel in the pores. Therefore, advanced modeling approaches, such as pore scale Lattice-Boltzmann (LB) chemotaxis models, have been proposed by Hilpert (2005) and Long and Hilpert (2007), and should be employed to simulate the experimental data along with address the limitations of previous modeling approaches.

In this study, we observed enhancement of contaminant mixing due to bacterial random motility. However, it would be interesting to see the effect on mixing enhancement due to bacterial chemotaxis, which is likely to prevail in groundwater contamination scenarios. Chemotaxis increases average bacterial run lengths and reduces tumbling frequency. Due to increased run lengths bacteria may encounter greater Knudsen diffusion in constricted porous media. The effects of these factors on contaminant mixing enhancement remain to be seen.

For the sake of simplicity in terms of device fabrication, data collection, and analysis, an evenly distributed well defined pore structure was chosen in all of the microfluidic devices used in this study. However, it is rare to find a systematic arrangement of pore structures in the real
world. Therefore, experimentation with the random arrangement of pore structures would be helpful to make use of the data in real world applications.

Loss of bacterial viability due to toxicity in this study is based on observation of a compromised cell membrane, which may not necessarily represent ultimate cell death. Upon re-exposure to favorable conditions, the cells may regain their viability. Also, under chemical stress cells may undergo various changes including gene changes that may result in changes to their reproduction rate as well as contaminant degradation potential. The contaminant concentrations used in this study were above saturation; however, lower contaminant concentrations may be of greater interest from the perspective of biodegradation. These issues need to be studied in further detail to further understand the long term goals of overall bioremediation processes.
List of References


Figure A.1: Schematic representing the bi-layer micro-fluidic device (MFD) used for testing transverse chemotactic migration of bacteria in dual-permeability porous media. The device design is similar to that presented in Figure 3.1 except the pore arrangement was modified to represent dual-permeability region. Cross-sections representing sampling locations at 4.9, 9.8, and 19.6 mm downstream from the attractant inlet point into the porous channel are also shown (top). The other specific pore geometry details are given in Figure 4.1 and Table 4.1.
**Figure B.1:** Comparison of concentration profiles of blank (no TCE) and TCE-containing plug assay experiments at different time intervals. Panels in rows 1 and 2 represent live cells (stained green) in blank and TCE-containing samples, respectively. The change in normalized concentration profile patterns of live cells (row 2) over the course of an experiment with TCE-containing samples in contrast with the nearly uniform live cell concentration profiles (row 1) in blank samples suggests a chemotactic response of *P. putida* F1 toward TCE. Toxicity effects of TCE can be seen by comparing row 3 (blank sample) and row 4 (TCE-containing plug). TCE diffusion into the bacterial chamber induces an accumulation of dead cells (stained red) over the course of an experiment, and is evident row 4, but not in row 3. For the purpose of comparison, concentration profiles are plotted on similar scales for both blank (row 1) and TCE-containing (row 2) samples for live cells and similarly for the dead cells (rows 3 and 4).
APPENDIX-C: COATED MICROFLUIDIC DEVICE: A BIOCHEMICAL SENSOR FOR TRACKING BACTERIAL AND CONTAMINANT TRANSPORT

An easy and popular method of coating sensor molecules on the surface is to use self-assembling properties of alkane chain molecules with thiol (-SH) groups on gold surface or Silanes (-SiOX) on silicon substrates (Raiteri et al 2001). The alkane chain molecules spontaneously form a uniform, densely packed, compact monolayer on the surface (Raiteri et al 2001) with another side of the molecule freely open to react with the sensor molecule. For this study, we adopted this as one of the methods for coating the devices with different sensor molecules for sugars or toluene concentration detection. We have used various amine reactive probes such as FluoReporter® FITC protein labeling Kit (Molecular probe, Invirogen), Dextran Cojugate Dye Texas Red® (Molecular probe, Invirogen), and pH indicator dyes FITC, Oregon Green, and SNARF SE (Molecular probe, Invirogen) etc for coating as sensor molecules on the device surfaces. Here, we present the methodologies and observations related to pH sensitive FITC dye coating for as an example, along with similar methods that were used for other sensor molecules as well. Silane (SiOX) group was chosen as a cross-linker between the device surface and the sensor molecules because the surface chemistry of the microfluidic devices (i.e. PDMS and glass) are similar to that of silicon.

C.1 FITC labeling of microfluidic devices

The labeling of FITC sensor on the MFD/glass surface consists of two steps:

**Step 1: Attaching amine group to the MFD/glass surface**

1. Glass surface was cleaned with acetone and DI water and then air dried at room temperature.
2. MFD/glass surfaces were exposed to oxygen plasma for 30-60 secs.
3. A 2% solution of 3-aminopropyltriethoxysilane (APTES) was prepared with acetone and the plasma treated surfaces of MFD/glass were submersed in it for a maximum up to 2 hours within 30 minutes of plasma treatment. Samples were taken out and rinsed in acetone and then DI-water and finally air dried. (Concentrations of APTES were varied between 1% to 10% and the exposure time was varied between 15 min to 2 hours). This produces should covalently bind amino groups on the glass surface.

**Step 2: Labeling with FITC dye**

4. Stock solution of FITC dye was prepared with glutaradehyde and diluted to different concentrations of working solutions. (Dye concentrations ranges from 100 µM to 2000 µM were explored).

5. MFD/glass surfaces were immersed in the different concentrations of dyes for varying lengths of times (30 min to 2 hours).

6. Coated surfaces were rinsed with DI water and air dried at room temperature and were prevented from direct light exposure.

7. Dried samples were observed under fluorescent microscope with appropriate excitation/emission filter. Images were captured and fluorescent intensity was measured using the ImageJ imaging tool.

**Observations:**

Figure C.1 shows the pH dependence of DL-aspartic acid (a chemoattractant for *E. coli* HCB33) concentration. A linear relationship was observed between pH and concentration around the optimal chemoattractant concentration range (i.e. 20 mM, as determined in chapter 4). For this reason, the pH sensitive FITC dye was chosen as a sensor molecule so that changes in the sensing signal could be directly correlated to the concentration of DL-aspartic acid. Figure C.2 shows a FITC sensor coated microfluidic channel under three different experimental conditions.
The coated channel was connected to the syringe pump and 10 % PBS was injected into the channel at the volumetric flow rate of 100 µl/h. The image in the left shows the coated MFD channel saturated with PBS before staring the flow and the images in the middle and right are images taken at 5 and 10 min after the flow was started in the device. It can be observed that the grey intensity rapidly diminishes as the PBS flows through the channel. This observation indicates a loose contact of the dye on the MFD surface instead of the predicted strong covalent bond as the dye was washed away with the buffer solution. Therefore, such loosely bounded dye could not be used for the intended chemical sensing. Similar observations were more prominent in most of the sensing dyes used for testing.

C.2 Concanavalin A and Glycogen coating

An alternative approach based on reversible binding instead of covalent bonding, purposed by Lvov et al 1995, was used for coating glass surfaces to develop sugar sensors. The stimuli-sensitive multilayer film was prepared by alternative deposition of Concanavalin A (Con A) and glycogen using the method presented by Soto et al 2004. This multilayer of ConA and glycogen film is sensitive to sugars due to the reversible bonding between Con A and D-glucose unit in the glycogen. Con A polymer contains four identical binding sites for sugar molecules (Sato et al 2005a). Due to the preferential binding of different sugar molecules to the Con A sites, which depends on the type of sugar and its concentration, the disintegration of the deposited multilayer is possible (Sato 2005b). Disintegration of the coated multilayer has been demonstrated for various sugars such as D-glucose, D-manose, methyl α-D-mannopyranoside and methyl α-D-glucopyranoside (Sato et al. 2004, Sato et al. 2005a, Sato et al. 2005b) though the rate of disintegration varied for different sugars. We have used this method for coating glass surfaces and studied disintegration of the coated layers following method proposed by Sato et al
2004 with appropriate modifications. Only the glass surface was coated in this method instead of the entire device.

![Graph](image)

**Figure C.1:** pH dependence of DL- aspartic acid concentration. Error bars indicates standard deviation of triplicate experiments.

![Images](image)

**Figure C.2:** FITC sensor coated microfluidic channel at three different experimental conditions. The vertical column in the middle of the image shows the coated portion of the channel and the
average intensity values at the top represents the average grey intensity of the coated in the respective image.

**Stock solution preparation:**

**Concanavalin A (Con A) solution preparation:**

1. A stock solution (2.5 mg/mL) of Con A with 0.1 M sodium bicarbonate buffer (approximate pH=8.3) was prepared as per manufacturer’s (Invitrogen) recommendation. (2 mL buffer in 5 mg Con A).
2. 1.0 ml of this stock solution was diluted to 23 ml with 0.1 M Tris-HCL buffer solution, and 3.15 mg MnCl₂ and 2.77 mg CaCl₂ was added.
3. The pH of this solution was adjusted to approximately 7.4 and the final volume was made up to 25 mL with 0.1 M Tris-HCL buffer solution.

**Glycogen solution preparation:**

1. 2.5 mg of glycogen was added in 25 mL 0.1 M Tris-HCL buffer solution with pH adjusted to approximately 7.4.

**Coating Procedure:**

1. Glass slide was soaked in Piranha solution (1:1 mixture of Sulfuric Acid and Hydrogen Peroxide) for 15-20 min to remove any organic contaminant from the surface. Slide was removed and rinsed with DI water three times.
2. Slide was dried with a nitrogen gas stream and finally heated to 125 °C to ensure complete removal of water.
3. 10% solution of dichlorodimethylsilane solution in toluene was made and the dried slide was soaked overnight at room temperature to make the surface hydrophobic. Washed with toluene, acetone and DI water.
4. Silylated glass slide was immersed in a Con A solution prepared above for 30 min at room temperature to deposit a layer through a hydrophobic force of attraction. Rinsed with DI water for 5 min to remove any weakly bound Con A.
5. Slide was immersed into glycogen solution for 30 min to deposit the polymer through an affinity between Con A and sugar residue in the glycogen. Finally the slide was rinsed in buffer solution.
6. Slide was observed under fluorescence micro-/stereoscope with appropriate filter. Images were taken and ImageJ was used to analyze the average grey intensity of the coated layer.
7. Steps 4 to 6 were repeated for multiple layer deposition.

8. For the sake of technology development disintegration of the coated layers was studied by submersing the coated sample into 50 mM solution of D-glucose for various lengths of times and measuring intensity as described above (bullet 6) after each time interval.

**Observations:**

There was no significant trend in the average grey intensity value with the addition of each new layer and the intensity remained nearly constant (Figure C.3). Similarly no trend was observed during disintegration experiments and the marginal intensity changes were almost random with both sugar and control experiments. These results may be due to the lack of sensitivity of the detection method used in this study i.e. fluorescent measurement. The previous studies used this method (Sato et al. 2004, Sato et al. 2005a, Sato et al. 2005b) have used spectroscopic measurement of the layer deposition and disintegration, which was not accessible in our lab.

![Figure C.3](image_url)

**Figure C.3:** Change in average grey intensity value with multiple layers of Con A and glycogen addition on the glass surface and with disintegration of layers at various time of exposure in experiment with sugar (50 mM D-Glucose) and no suger (control). Error bars indicates standard deviation of triplicate experiments.
APPENDIX-D: EXPERIMENTAL PROTOCOLS

D.1 Micro Fabrication

D.1.1 Photolithography process for a SU8-2025 mold fabrication for MFDs

Instruments and other supplies required:
1. Specialty Coating System Inc., G3P-8 Spin Coat (Spin coater)
2. Karl Suss MJB-3 (UV light exposure)
3. Photoresist SU-8 2025
4. Wafer/Substrate- 3” silicon wafer
5. Photomasks
6. SU-8 developer/IPA/DI water
7. Hot Plate(s), Syringes/Droppers/pipette+tips, Timer, Wafer tweezers, Surface thermometers, Cover glass to cover photo-mask- may be SU-8 coated, Syringe needles, Scissor, Petri dish/developing bowl, Instructions

Wafer Pretreatment – depends on the quality. If ordered good quality pretreatment may not required. Rinse with Acetone/Isopropanol /DI water. Dehydrate 125°C for 5 minutes.

SU8 Coating
Desired thickness - 20 μm
Static dispense 3 ml photoresist (1 ml per inch of diameter)
Spread cycle: Ramp to 500 rpm @ 100 rpm/sec, hold for 5-10 seconds, ramp to final spin speed of 1725 rpm @ 300 rpm/sec, hold for 30 seconds

Soft Bake
Pre-bake 1.5 minute @ 65°C
Soft-bake 6 minutes @ 95°C

Expose (UV 350-400 nm; 365 nm recommended)
Total energy dose is 160 mJ/cm²
Exposure time = total energy dose/ rated power on equipment
Exposure time = 160/ 7.5 = 21.3 sec

Post Exposure Bake
1 minute @ 65°C
6 minute @ 95°C

Develop
Immersion develop for 5 minute in SU-8 Developer

Rinse & Dry
Spray and wash with fresh solution of SU-8 developer followed by spray/wash with IPA
D.1.2 PDMS softlithography process for MFD fabrication

The PDMS microchannel is molded over the microfabricated SU-8 mold in the previous section (section D.1.1), heat cured, and then released from the mold. The PDMS channel is then permanently bonded to a thin glass slide for using/viewing under the microscope. Following are the specific step of the process

- Micromold Silane Deposition
  - The silane prevents the cured PDMS from adhering to the thin substrate.
  - Make sure that the SU-8 mold on the silicon substrate is in good condition. Clean if required using acetone, IPA, DI-water, and allow substrate to dry. Scotch® tape can be used sometime to clean dust from the surface.
  - Place one drop of silane in opaque container in vacuum deposition chamber.
  - Place the mold in a petri dish and put it in the deposition chamber with cover open.
  - Vacuum for 30 mins to allowing the silane to go to vapor phase. Turn off the pump and let the chamber be at vacuum for another 30 min to let the silane deposition on the mold surface.

- PDMS Elastomer Preparation (This step can be proceeded during the deposition step)
  - Get a clean 50mL plastic disposable container/ weighing dishes.
  - Place the container on a 1/10\textsuperscript{th} gram accuracy scale, and tare to zero.
  - For one 3‘ wafer mold, add ~12 ml PDMS resin to the container using a 10mL syringe.
  - Take a 1mL syringe and add ~1.5 ml PDMS curing agent to the glass container to make the ration 8:1.
  - Use smooth glass rod to blend the PDMS mixture thoroughly. This should take about 3-5 minutes.
  - Once the PDMS is thoroughly mixed, it must be degassed using another vacuum chamber. Do not use the similar vacuum chamber that was used for silane deposition. As vacuum increases, the PDMS will want to bubble and overflow out of the top of the container, do NOT let it. Use paper towel below the container for the safety purpose.
  - Turn off the vacuum pump once the bubbles push past the top edge of the jar, and restart once they have receded approximately 5 minutes later. Eventually the bubbles will become large enough where almost all of them will pop at once.
  - Allow for the PDMS to fully degas under full vacuum till all the bubbles are removed, usually the process can take 30 min to 60 min.
  - This process can be achieved even without using vacuum but may take several hours.

- Heat Curing
  - Open the silane coating chamber inside the chemical fume hood and let the vapor come out for five mins.
  - While waiting for the above process to complete, set the hot plate @ 45-50 °C.
  - Takeout the silane coated petri dish, contain mold, and place it on the hot plate.
  - Pour the PDMS mixture gently into the mold to avoid any bubble formation and especially try to avoid bubbles around the structures areas.
  - Cover the petri dish and let the bubble pop out. Slow heating help releasing bubbles quickly by reducing the viscosity of the PDMS mixture. PDMS mixture can be gently heated, not more than 40 °C, before the degassing step to help reducing the degassing time.
  - Once all the bubbles come out ramp the temperature to 90 °C and allow to bake for 1 hour. Turn off the hot plate after PDMS mixture is solidified and let it cool.
• Glass Slide Preparation
  o Take one 43x50 glass slide (or as per the requirement of the structure size on the mold), and rinse it with IPA and DI-water.
  o Clean the slide with heptane as well, using a q-tip.
  o Rinse the slide three times with DI-water, and dry with N₂.
  o Sometime this cleaning step is not required if better quality slides are being used. Just dusting with nitrogen is enough.

• De-Molding PDMS and Punching Inlets and Outlets
  o Take sharp razor and carefully cut into the solidified PDMS layer to remove the desired structure printed on the PDMS layer.
  o Carefully take the patterned PDMS out of the petri dish and place it on a clean surface, may be another clean/new petri dish with up-side down i.e. printed pattern should remain at the top
  o Trim the edges of this PDMS layer into the desired shape, if required. It is sometimes helpful to cut edges in such a way that the ultimate contact angle between the PDMS and glass is < 90° i.e. a “hydrophilic” looking angle.
  o Take sharp punching needle of desired gauge size and push firmly through the PDMS at the locations where an inlet or outlet tubing are intended to be inserted. Make sure to have firm support behind the PDMS layer to avoid developing cracks around the punching area.
  o Lift the PDMS into the air using the needle punch.
  o Take a smaller syringe needle and push the PDMS plug out of the needle punch.
  o Wipe away the plug and repeat it for all other inlets/outlets.

• Plasma Treatment
  o Place glass slide (clean side up) and punched PDMS layer (patterned side up) on the quartz stage inside the plasma cleaner.
  o Close and vacuum down the plasma chamber for 10-15 mins at least. Make sure the proper vacuum inside the chamber which can be read on the attached pressure gauge (less than -29 PSI).
  o Make sure the valve is in the closed position for a vacuum seal.
  o Once the chamber has pumped down, using the left trigger, turn the chamber on.
  o Plasma treat for 30-45 seconds by turning the dial to the right to HIGH. An UV glow of the plasma discharge should be visible with violet color.
  o After treatment, release the valve to remove the vacuum pressure.

• Bonding
  o Take the quartz stage out of the plasma chamber and lay it on the table.
  o Quickly take the PDMS and flip is over onto the glass slide
  o Press down gently to remove all air bubbles and help bonding. Be very careful around inlet and outlet areas where undesired surfaces are prone to bond with the glass surface. Do to press around these areas.
  o The bonded device can be put inside an oven set at around 60-70 °C to further strengthen the bonding but this step is not necessarily required.
  o Set inside petri dish for at least 10 minutes, after which the bond can be tested by trying to lift a corner. The PDMS should not lift at all.

• Attaching tubes.
  o Cut length of tube.
  o Push the cut end into the hole in the PDMS.
D.2 Live/Dead Bacterial Viability Assay

Instruments and other accessories required:

1. Centrifuge
2. Fluorescence Microscope equipped with adequate filter sets listed in Table 1 of Molecular Probe’s product information.
3. 2-mL glass vials with PTFE rubber septa
4. Spectrophotometer with glass or acrylic absorption cuvettes (1 cm path length)
5. Slides and cover slips
6. Vortex

Reagents:

1. 0.85% of NaCl Buffer (Dissolve 0.85 g NaCl in 100 mL water)
2. 70% isopropyl alcohol
3. Molecular Probes Viability Kit: L7012 SYTO 9 dye, 3.34 mM (Component A) and Propidium iodide, 20 mM (Component B)

Phosphate wash buffers are not recommended for washing because they appear to decrease staining efficiency.

Preparation of Bacterial Suspensions for staining

1. Take 15 mL of the Pseudomonas Putida F1 culture suspension, from exponential growth phase (0.7-1.0, 1 cm OD @ 590), in a 15 mL centrifuge tube and concentrate by centrifugation @ 5000 rpm for 10–15 minutes.
2. Remove the supernatant AND resuspend the pellet, by gently vortexing/shaking, back to 15 mL with 0.85% NaCl buffer and again centrifuge @ 5000 rpm.
3. Resuspend (gently) and dilute to OD 0.15 @ 670 with 0.85% NaCl buffer, around 2 mL per sample.
4. Take 2 mL of bacterial suspension from step 1.3, in a 2 mL glass vial and add 3 ul of 1:1 solution of dye mixture. Vortex briefly and leave for staining for 15-20 min in dark.
5. Briefly vortex/shake the sample again and crimp under the hood. Inject desired amount stressor (TCE, Toluene etc..) in the sample using gas tight syringe (Hamilton Series 800)
6. Withdraw small amounts (10-15 uL) of aliquots at defined time intervals from sealed vials after mixing well by vortexing or shaking.
7. Deposit the withdrawn sample onto the microscopic slide and observe under fluorescence microscope.
8. Take five images, each with green and red filters, at five randomly selected locations.
9. Count green (live) and red (dead) bacteria in each images.
APPENDIX-E: HYDROGEOLOGIC CHARACTERISTICS OF THE MICROFLUIDIC DEVICES

Table E.1: Hydrogeological properties of the MFDs used in this thesis

<table>
<thead>
<tr>
<th>Device Type</th>
<th>Channel height (mm)</th>
<th>Channel width (mm)</th>
<th>Porosity</th>
<th>Darcy's Velocity (m/day)</th>
<th>Darcy's Velocity (mm/s)</th>
<th>Permeability $k \times 10^4$ (mm$^2$)</th>
<th>Peclet Number</th>
<th>Reynolds Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFD I</td>
<td>0.02</td>
<td>6.30</td>
<td>0.42</td>
<td>9.59</td>
<td>0.11</td>
<td>1.36</td>
<td>1449</td>
<td>0.03</td>
</tr>
<tr>
<td>MFD I</td>
<td>0.02</td>
<td>6.30</td>
<td>0.42</td>
<td>19.19</td>
<td>0.22</td>
<td>1.36</td>
<td>2897</td>
<td>0.07</td>
</tr>
<tr>
<td>MFD I</td>
<td>0.02</td>
<td>6.30</td>
<td>0.42</td>
<td>38.38</td>
<td>0.44</td>
<td>1.36</td>
<td>5794</td>
<td>0.13</td>
</tr>
<tr>
<td>MFD I</td>
<td>0.02</td>
<td>6.30</td>
<td>0.42</td>
<td>76.76</td>
<td>0.89</td>
<td>1.36</td>
<td>11588</td>
<td>0.27</td>
</tr>
<tr>
<td>MFD II</td>
<td>0.02</td>
<td>6.30</td>
<td>0.36</td>
<td>9.59</td>
<td>0.11</td>
<td>0.44</td>
<td>1159</td>
<td>0.03</td>
</tr>
<tr>
<td>MFD II</td>
<td>0.02</td>
<td>6.30</td>
<td>0.36</td>
<td>19.19</td>
<td>0.22</td>
<td>0.44</td>
<td>2318</td>
<td>0.07</td>
</tr>
<tr>
<td>MFD II</td>
<td>0.02</td>
<td>6.30</td>
<td>0.36</td>
<td>38.38</td>
<td>0.44</td>
<td>0.44</td>
<td>4635</td>
<td>0.13</td>
</tr>
<tr>
<td>MFD II</td>
<td>0.02</td>
<td>6.30</td>
<td>0.36</td>
<td>76.76</td>
<td>0.89</td>
<td>0.44</td>
<td>9270</td>
<td>0.27</td>
</tr>
<tr>
<td>MFD III</td>
<td>0.02</td>
<td>6.40</td>
<td>0.36</td>
<td>9.45</td>
<td>0.11</td>
<td>0.68</td>
<td>1141</td>
<td>0.03</td>
</tr>
<tr>
<td>MFD III</td>
<td>0.02</td>
<td>6.40</td>
<td>0.36</td>
<td>18.89</td>
<td>0.22</td>
<td>0.68</td>
<td>2281</td>
<td>0.07</td>
</tr>
<tr>
<td>MFD III</td>
<td>0.02</td>
<td>6.40</td>
<td>0.36</td>
<td>37.78</td>
<td>0.44</td>
<td>0.68</td>
<td>4563</td>
<td>0.13</td>
</tr>
<tr>
<td>MFD III</td>
<td>0.02</td>
<td>6.40</td>
<td>0.36</td>
<td>75.56</td>
<td>0.87</td>
<td>0.68</td>
<td>9126</td>
<td>0.26</td>
</tr>
<tr>
<td>*MFD IV</td>
<td>0.02</td>
<td>6.30, 0.36</td>
<td>0.42</td>
<td>4.80</td>
<td>0.06</td>
<td>1.36, 0.47</td>
<td>1158, 1448</td>
<td>0.03, 0.03</td>
</tr>
<tr>
<td>*MFD IV</td>
<td>0.02</td>
<td>6.30, 0.36</td>
<td>0.42</td>
<td>9.59</td>
<td>0.11</td>
<td>1.36, 0.46</td>
<td>2317, 2897</td>
<td>0.05, 0.07</td>
</tr>
<tr>
<td>*MFD IV</td>
<td>0.02</td>
<td>6.30, 0.36</td>
<td>0.42</td>
<td>19.19</td>
<td>0.22</td>
<td>1.36, 0.45</td>
<td>5794, 4635</td>
<td>0.11, 0.13</td>
</tr>
</tbody>
</table>

*Two values reported for MFD-IV are for low and high permeability regions in that order.
Rajveer Singh was born in Uttarkashi, UP, India. He received his undergraduate degree in Agricultural Engineering from Govind Ballav Pant University of Agriculture and Technology, Pantnagar, UA, India in 2002. After that he joined Environmental Engineering and Management programme at Indian Institute of Technology, Kanpur, India and received his master’s degree in 2004. After briefly working as ‘Senior Research Associate’ at Indian Institute of Technology, he joined Ph.D. in Environmental Engineering at Drexel University in 2006. His research interests spans in the areas of Environmental micro-fluidics; In-situ bioremediation and transport of contaminants in groundwater aquifers; Advective, dispersive, and chemotactic transport of bacteria in saturated porous media; Contaminants toxicity to contaminant-degrading bacteria; Water and wastewater treatment; Surface and subsurface hydrology.

Awards and Honors:

- Winner of 2010 Graduate Student Award in recognition of outstanding potential for future contribution to the field of Environmental Chemistry- American Chemical Society, Division of Environmental Chemistry.
- George Hill, Jr. Fellowship award for maintaining excellent academic performance - Drexel University, 2009.
- Freshmen Engineering Design Fellowship for teaching and leadership skills - Drexel University, 2008-2009.

Selected peer reviewed publications:

- Singh, R., and Olson, M.S. “Transverse chemotactic migration of bacteria from high to low permeability region in a dual-permeability porous microfluidic device”. (Manuscript in preparation for peer review publication).