Isolation and Study of Bacteria Using Physical Constrictions

A Dissertation Presented

By

Nil Tandogan

to

The Department of Chemical Engineering

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

In the field of

Chemical Engineering

Northeastern University

Boston, Massachusetts

May, 2016
To my Mom, for being an amazing role model...
ACKNOWLEDGEMENT

I couldn’t have accomplished this journey without the amazing people in my life who were always supportive and by my side whenever I needed help and courage.

I would like to initially thank my advisor, Prof. Edgar D. Goluch for his guidance, continuous support and patience throughout my PhD. I am very grateful for the opportunity to work in his lab on a project that I truly enjoyed. I would like to also thank him for teaching me the importance of networking and participating in conferences and professional events. It has helped me tremendously in improving my professional skillsets.

I would like to thank my PhD committee members, Prof. Anand Asthagiri, Prof. Shashi Murthy, and Prof. Slava Epstein for their time, contribution, and guidance to my research. I greatly appreciate your valuable feedback regarding my research and progress.

I would like to thank Dr. Thaddaeus Webster, Dr. Pegah N. Abadian, and Hunter Sismaet for being the most amazing lab mates that anyone could ask for. It was a privilege to work with you all, and call you as my friends. Thad, thank you for patiently teaching me everything you know about microfabrication when I first joined the group. I always enjoyed our coffee runs, discussions about research, politics and life. Pegah, thank you for all the collaborations and discussions we have done together, being my roommate at conferences, and all the conversations we had in Turkish. Hunter, thank you for always supporting me, and being an awesome friend. I will greatly miss our conversations, your mini surprises, and our collaboration. Hopefully, I am a more organized person now thanks to you.
I would also like to thank all the undergraduate students who worked with me and contributed to this research, especially to Richard Crowley, Yaxing Amy Zhu, Bowen Huo, Abigail Paglia, and Owen Porth.

I would like to thank Northeastern University, Department of Chemical Engineering for giving me the opportunity to pursue my PhD for the past five years, and the NSF for funding part of this research.

I would like to acknowledge Scott McNamara and David McKee at Kostas Nanoscale Technology and Research Center for their continuous help and support in the cleanroom, and for sharing their extensive knowledge in microfabrication processes with me.

I would like to acknowledge William Fowle from Department of Biology for teaching me how to use electron microscopy instruments, and sharing his expertise in SEM and TEM.

I would like to acknowledge Patricia Rowe, Jessica Smith-Japhet for helping me with the administrative related issues, and Robert Eagen for always helping me whenever I needed help, and for building tools essential for my research.

I would like to thank my classmates and close friends, Dinara Andirova and Avinash Kola, for making Boston feel like home. This dissertation wouldn’t be possible without your support.

Last but not least, I would like to express my sincere thanks and gratitude to my family: To my mom, Måkbule Tandogan, my dad, Mustafa Tandogan, and my brother, F. Okan Tandogan. I always feel your unconditional support, love, encouragement, and
motivation. No matter where you are and what time it is at home, I know that you are only a phone-call away. I am very grateful to have you.
This dissertation describes the development of new devices and techniques for the isolation and study of bacterial cells. In aim one, a conceptually simple and effective polymer microfluidic device with sub-micrometer constrictions was used to isolate individual bacterial species from complex mixtures. To demonstrate this principle, a heterogeneous culture with two model microorganisms, *Pseudomonas aeruginosa* and *Escherichia coli*, were successfully separated from one another. Out of 49 chambers tested, 22 chambers contained species, and 7 chambers had multiple species. Furthermore, 16 out of 49 chambers did not show any bacterial growth because the constrictions were blocked by dust or air bubbles. It was also observed that as the constriction width reaches 2.5 µm and above, the separation yield decreases. Two *Escherichia coli* strains that were labeled with different colors of fluorescent protein were also sorted to show that bacterial cell isolation inside the device is random.

In the second aim, polycarbonate and aluminum oxide membranes were integrated onto polymer microfluidic devices to optimize the system for *in-situ* isolation and cultivation of species from the environment and human fecal samples. The new design enabled isolation chambers within the devices to communicate with the environment through the nanopores in the membranes. The device therefore bypasses the difficulties that occur when cultivating low-abundance and slow growing species from the environment.

The third aim focuses on bacterial behavior in confinements under applied pressure, which is critical in water filtration processes. For this purpose, two model normal and pathogenic microorganisms were used to obtain pressure profiles and
understand how bacteria move through porous media and how bacterial behavior changes with respect to species physical characteristics, pore size, and applied pressure. This study provided important insight into the effectiveness of water filtration processes.

The designed sub-microfluidic devices are very versatile that they could also be used to monitor bacterial behavior as the cells are exposed to chemicals. In the fourth aim, initial studies were conducted to investigate the antibiotic susceptibility of bacteria in real-time by coupling a microfluidic device to a Surface Plasmon Resonance imaging (SPRi) system. As a proof-of-concept, *Staphylococcus aureus* culture was first exposed to penicillin/streptomycin cocktail inside a microfluidic device, and significant changes in cellular morphology were observed. Then, the gold surface needed for the SPRi system was chemically functionalized with a thiol-silane linker, providing a robust bonding between the PDMS device and the gold surface of the prism to perform the studies. This protocol will provide a new direction to monitor biofilm removal in microfluidic devices in real-time using SPRi technology.
TABLE OF CONTENTS

TABLE OF CONTENTS ........................................................................................................... viii
LIST OF FIGURES .................................................................................................................... x
LIST OF TABLES ......................................................................................................................... xvii
1.0 Introduction ......................................................................................................................... 1
2.0 Critical Literature Review ................................................................................................. 5
  2.1. Single Cell Studies ....................................................................................................... 5
    2.1.1. Micro- to Nano-Scale Systems ............................................................................. 5
    2.1.1.1. Single Cell Isolation ....................................................................................... 5
    2.1.1.2. Bacterial Cell Cultivation ............................................................................. 9
    2.1.2. Droplet-Based Cell Capturing and Cultivation Methods ...................................... 14
    2.1.3. Electrophoretic Separations, Optical Tweezers and Other Techniques .................. 18
      2.1.3.1. Electrophoretic Separation Techniques ...................................................... 18
      2.1.3.2. Optical Tweezers ....................................................................................... 22
      2.1.3.3. Other Techniques ....................................................................................... 23
  2.2. Bacteria ......................................................................................................................... 24
    2.2.1. Gram-Negative and Gram-Positive Bacteria ....................................................... 24
      2.2.1.1. Chemotaxis ............................................................................................... 25
      2.2.1.2. Quorum Sensing and Biofilms .................................................................... 30
      2.2.1.3. Antibiotic Susceptibility .......................................................................... 34
    2.2.2. Uncultivable Bacteria ......................................................................................... 37
  2.3. Metagenomics ............................................................................................................... 42
  2.4. Bacterial Behavior and Biomechanics ......................................................................... 45
  2.5. Effect of Fluid Flow and Shear Stress on Bacteria .................................................... 47
  2.6. Microfluidic Devices and Nanofabrication Techniques .............................................. 49
    2.6.1. Microfabrication of Glass/Si and Etching Techniques ........................................ 49
    2.6.2. Lithography Techniques ..................................................................................... 52
      2.6.2.1. Electron Beam Lithography ....................................................................... 52
      2.6.2.2. Photolithography ....................................................................................... 53
      2.6.2.3. Soft Lithography ....................................................................................... 54
  3.0 Research Aims ................................................................................................................... 58
  4.0 Methodology ..................................................................................................................... 61
  4.1. Aim 1 .............................................................................................................................. 61
    4.1.1. Design and Fabrication of the Master Wafer ...................................................... 61
    4.1.2. PDMS Devices ................................................................................................. 66
    4.1.3. Bacterial Cultures ............................................................................................. 67
    4.1.4. Experimental Procedure and Fluorescence Imaging .......................................... 68
    4.1.5. Computational Modeling of the Device ............................................................. 70
  4.2. Aim 2 .............................................................................................................................. 70

viii
LIST OF FIGURES

Figure 1: Schematic of the microfluidic device. Main entrance is connected to isolation chambers via nano- and sub-micron constrictions. Heterogeneous bacterial culture that enters the main entrance self-sort into different isolation chambers with the help of chemotaxis and size-specific constrictions. ................................................................. 4

Figure 2: Wet etching process of the chromium metal with a Chromium etchant. The etching occurs anisotropically. The photoresist on top of the chromium metal acts as a mask to maintain the height of the chromium, and was removed after etching process was completed. .......................... 51

Figure 3: Chemical formula of Poly-dimethylsiloxane (PDMS). ........................................ 56

Figure 4: A master wafer design drawn with Tanner L-Edit. It includes 8 different microfluidic chips with large isolation chambers and a main entrance on a 3-inch Si wafer. (Constrictions are not visible at this magnification). .......................................................................................... 62

Figure 5: (A, B) SEM images of a completed wafer. (A) One large isolation chamber is connected to a sub-micron constriction. (B) The main entrance is connected to 3 isolation chambers via square and circular constrictions. .......................................................................................... 63

Figure 6: (A, B) Brightfield images of the master wafer containing different constriction designs. (A) A straight constriction. (B) A tapered constriction design. ...................................................................................... 63

Figure 7: Schematic of a PDMS microfluidic device fabrication. 1) PMMA is spun on a 3 inch Si-wafer. 2) The constriction pattern is created with electron beam lithography and developed. 3) Chromium is sputtered (height of the constriction is determined by this step). 4) Lift-off is done. 5) Positive photoresist is spun. 6) Large features are aligned over the constrictions and created with photolithography. 7) Large features are developed, completing the master wafer. 8) PDMS is molded. 8) PDMS is peeled off, access holes are drilled. 9) The device is bonded to a glass cover slip using oxygen plasma (adapted from ref [250]). ...................................................................................... 65

Figure 8: Photograph of a completed PDMS sub-microfluidic device. ....................................................... 67

Figure 9: (A-D): Experimental procedure for filling the PDMS microfluidic device. (A) All the isolation chambers were filled with LB while the main entrance was filled with PBS. (B-C) Bacteria were separately cultured on agar plates, and grown in 6 mL of growth media. (D) A bacterial culture was prepared by mixing two species and a droplet of this mixture was inoculated into the access hole of the main entrance. 69

Figure 10: (A) Schematic of the microfluidic device coupled with a nanoporous membrane. (B) The PDMS microfluidic device with a polycarbonate membrane covering all the access holes of the isolation chambers while leaving the main entrance exposed. ................................................................. 72

Figure 11: The PDMS sub-microfluidic device with an Anodisc membrane covering all the access holes of the isolation chambers while leaving...
the main entrance was incubated in a bottle filled with the river sample. The accumulation of bacteria on the membrane could be seen from the yellowish color change on top of the membrane. .......................... 75

Figure 12: (A-C) The setup for the electrochemical detection of pyocyanin using an electrochemical sensor. The PDMS well was filled with PBS solution and a droplet of pyocyanin was placed on top of the well when: (A) A PDMS well with no membrane (B) A PDMS well covered with a PC membrane. (C) A PDMS well covered with an Anodisc membrane. .......................................................................................................................... 78

Figure 13: (A) The PDMS microfluidic device. (B) The schematic of one part of the microfluidic device with a sub-micrometer constriction attached to the constriction. A precise pressure head was applied at the inlet using a microfluidic flow control system. The outlet channel is open to the atmosphere (adapted from ref [255]). .......................................................... 80

Figure 14: (A, B) SEM images of a constriction after treated with Chromium etchant (A) The constriction on the wafer (B) The constriction on the PDMS. .......................................................... 83

Figure 15: SEM images of one isolation chamber that is connected to the main entrance with a sub-micrometer constriction. (A, B) SEM images of the fabricated master wafer. Constrictions were fabricated with electron-beam lithography to obtain exact widths at high precision in a single step. (C, D) SEM images of the PDMS device. (D) The magnified image of the constriction shown in (C). White particles seen on the SEM images formed during the metal sputtering step of SEM sample preparation. Devices used in experiments do not have any visible particles on the surface (adapted from ref [250])............... 85

Figure 16: (A-C) Optical images of permanently bonded PDMS. (A) Uncollapsed constriction. (B) Partially collapsed constriction. (C) Fully collapsed constriction. .......................................................... 86

Figure 17: Isolation of CFP P. aeruginosa via a 950 nm tall, 2.5 µm wide, and 20 µm long constriction. (A) GFP filtered image of the device. CFP P. aeruginosa formed 2 lines inside the constriction. (B) RFP filtered image of the device. M-cherry E. coli could not enter the constriction or the isolation chamber (adapted from ref [250]). ............ 88

Figure 18: (A-D) Fluorescence images of CFP P. aeruginosa PAO1 and m-cherry E. coli in a device with 1.5 µm wide and 700 nm tall constriction. (A) 40X GFP filtered image. P. aeruginosa formed a single line in the constriction and reached the isolation chamber. (B) 100X GFP filtered image of the same constriction. (C, D) 40X and 100X RFP filtered images of the same device, respectively. The isolation chamber and the constriction in both images are dark due to the absence of E. coli while the presence of the bacteria can be seen in the main entrance (adapted from ref [250]). ..................... 89

Figure 19: (A-B) GFP and RFP filtered images of 2:1 (v/v and concentration) ratio of m-cherry E. coli : CFP P. aeruginosa in a device with 700 nm tall, 1.5 µm wide constriction, respectively. (A) CFP P. aeruginosa
entered the isolation chamber by forming a visible single file in the
constriction (B) m-cherry E. coli were present in the main entrance,
but their concentration was much lower than P. aeruginosa even
though their initial inoculum concentration was higher than
P. aeruginosa (adapted from ref [250]). .................................................. 90

Figure 20: (A-B) GFP and RFP filtered images of 1000:1 (v/v and
centrations) m-cherry E. coli : CFP P. aeruginosa in a device
with 950 nm tall, 1.75 µm wide constrictions, respectively.
(A) CFP P. aeruginosa entered and populated the isolation chamber
preventing E. coli from entering. P. aeruginosa in the main entrance
and in the constriction were not visible using fluorescence microscopy
due to photobleaching. (B) m-cherry E. coli were present in the main
entrance, but could not enter the isolation chamber (adapted from ref [250]). .......... 91

Figure 21: (A, B) Self sorting of GFP E. coli from m-cherry E. coli via a 950
nm tall, 1.5 µm wide, 40 µm long constriction. (A) GFP filter, 100X
magnification. A single column of GFP-labeled cells is visible. (B) RFP-
filter, 100X magnification. No RFP-labeled cells are visible in the
constriction or in the isolation chamber (adapted from ref [250]). .......... 94

Figure 22: Micrographs of (A) Psychroserpens sp. and (B) Roseobacter sp.
strains, respectively. ................................................................. 95

Figure 23: Separation of Roseobacter via a 950 nm tall, 3 µm wide constriction.
(A-B) 40X and 100X brightfield images from the same section of the
device. Roseobacter sp. was present both in the main entrance and the
isolation chamber whereas Psychroserpens sp. existed only in the main
entrance. (C) The brightfield image of individual Roseobacter sp. cells
in LB. The colonies were randomly picked from the plate shown in (D).
(D) The photograph of the culture plate containing only Roseobacter
sp. colonies. The sample was collected from the isolation chamber
shown in (A-B) and spreaded onto the LB-agar plate. ......................... 96

Figure 24: (A,B) The plots demonstrating the concentration of Leucine
(black color) and Serine (blue color) in the isolation chamber
versus time, respectively. The amino acids are diffusing from
the isolation chamber into the main entrance via a constriction
(adapted from ref [250]). ..................................................................... 98

Figure 25: SEM images of (A) An Anodisc membrane with 0.02 µm pore size
(B) A PC membrane with 0.05 µm pore size. Scale bar is 0.5 µm. ........... 99

Figure 26: The brightfield image of a negative control device with a PC
membrane on top of all the access holes. Both the isolation
chamber and the main entrance of the device did not show any
bacterial growth after 2 days of incubation at room temperature
in a bacterial culture. ......................................................................... 100

Figure 27: Cyclic voltammogram of 10 µM pyocyanin in 10 mM PBS from
-0.7 to 0.0 V at a scan rate of 0.5 V/s. Cathodic and anodic peak
currents can be obtained using a baseline-subtraction method to
determine the maximum currents of those peaks. Note: The polarity was set to anodic positive on the potentiostat.

Figure 28: Plot of peak current (A) vs. scan rate$^{1/2}$ (V/s)$^{1/2}$ of 10 µM Pyocyanin molecule in 10 mM PBS solution. The diffusion coefficient can be determined from the slope of the resulting lines using the Randles-Sevcik equation.

Figure 29: Effect of a nanoporous membrane covering the access hole of an isolation chamber in the diffusion of pyocyanin into the device. (A-C) COMSOL model showing the surface concentration of pyocyanin throughout the access hole after 10 minutes (A) without a membrane, (B) with a PC membrane, and (C) with an Anodisc membrane. (D-F) Plots depicting the empirical and computational data of pyocyanin concentration at the bottom of the access hole over time (D) without a membrane, (E) with a PC membrane, (F) with an Anodisc membrane (blue lines represent the computational data; black points represent the empirical data).

Figure 30: Measurement of pyocyanin concentration at the surface of the electrochemical sensor over time as it diffuses from the top of an open well with no membrane (black line). The well was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin. Theoretical models of 2D (gray line), and 3D transport, using either the experimentally determined ($6.10 \times 10^{-9}$ m$^2$/s) (red line) or an optimized diffusion coefficient ($9 \times 10^{-9}$ m$^2$/s) (blue line).

Figure 31: Measuring pyocyanin at the surface of the electrochemical sensor over time as it diffuses through a well that is covered with a polycarbonate membrane (black line). The well was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin on top of the membrane. Theoretical models of 2D (green line), and 3D transport using the experimentally determined ($6.10 \times 10^{-9}$ m$^2$/s) diffusion coefficient with multiple pores (red line) and with 1 pore (gray line), and 3D transport with an optimized diffusion coefficient ($9 \times 10^{-9}$ m$^2$/s) (blue line).

Figure 32: Measuring pyocyanin at the surface of the electrochemical sensor over time as it diffuses through a well that is covered with an Anodisc membrane (black line). The well was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin on top of the membrane. Theoretical models of 2D (gray line) and 3D transport using either the experimentally determined ($6.10 \times 10^{-9}$ m$^2$/s) with multiple pores (red line) and with 1 pore (green line), and a 3D transport with an optimized diffusion coefficient ($9 \times 10^{-9}$ m$^2$/s) (blue line).

Figure 33: A 2D theoretical model of pyocyanin concentration diffusing down through the isolation chamber to the surface of the electrochemical sensor after 10 minutes, using an open well with no membrane. The bottom rectangle represents the well, which was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin.
droplet of 10 µM pyocyanin (top rectangle). The experimentally
determined \((6.10 \times 10^{-9} \text{ m}^2/\text{s})\) diffusion coefficient was used in the
model. ................................................................. 110

Figure 34: A 2D theoretical model of pyocyanin concentration diffusing down
through the isolation chamber to the surface of the electrochemical
sensor after 10 minutes, using a well that is covered with a
polycarbonate membrane. The bottom rectangle represents the
well, which was prefilled with 100 µL of 10 mM PBS prior to
adding a 50 µL droplet of 10 µM pyocyanin (top rectangle). A
single pore between the solutions is used to simulate the active
transport area of the membrane. The experimentally determined
\((6.10 \times 10^{-9} \text{ m}^2/\text{s})\) diffusion coefficient was used in the simulation........ 111

Figure 35: A 2D COMSOL theoretical model of pyocyanin concentration
diffusing down through the isolation chamber to the surface of
the electrochemical sensor after 10 minutes, using a well that is
covered with an Anodisc membrane. The bottom rectangle
represents the well, which was prefilled with 100 µL of 10 mM
PBS prior to adding a 50 µL droplet of 10 µM pyocyanin
(top rectangle). A single pore between the solutions is used to
simulate the active transport area of the membrane.
The experimentally determined \((6.10 \times 10^{-9} \text{ m}^2/\text{s})\) diffusion
coefficient was used in the model. ................................................................. 112

Figure 36: (A-B) Taxonomic tree obtained from the Charles River.
(A) The winter samples (B) The summer samples. ................................. 116

Figure 37: (A-F) The 2D theoretical models of pressure drop in the
sub-microfluidic device as 100 mbars of pressure (red color)
was applied from each pressure inlet and the outlet was open
to atmospheric conditions (blue color). (A) The pressure profile
within the whole microfluidic device. (B) The pressure drop within
the constriction without any bacteria. (C) The pressure drop profile
as one bacterial cell is at the entrance of the constriction. (D) The
pressure drop profile as one bacterial cell gets squeezed inside the
constriction. (E) The pressure drop profile as two bacterial cells
are at the entrance of the constriction. (F) The pressure drop profile
as a bacterial cluster accumulates at the entrance of the constriction.... 120

Figure 38: Images of 1 µm diameter beads passing through 950 nm tall
constrictions with varying widths. (A-B) 20X, brightfield and
GFP filter images of beads that entered 5 µm wide constrictions
at 1.6 bars of pressure, respectively. (C, D) 40X, brightfield and
GFP filter images, respectively. Beads entered 2.5 µm wide
constrictions at 2.3 bars of pressure. (E, F) 40X, brightfield and
GFP filter images, respectively. Only 1-2 beads were seen inside
the entrance of 1.5 µm wide constrictions with 3.4 bars of applied
pressure (adapted from ref [275])................................................................. 122

Figure 39: (A-B) The SEM images of E. coli cells.............................................. 123

Figure 40: (A-B) The SEM images of P. aeruginosa cells.............................. 123
Figure 41: Applied pressure change needed for *E. coli* cells to enter 950 nm tall constrictions with varying widths. # indicates sample size is 2 for that constriction. Error bars indicate standard deviation. 126

Figure 42: Applied pressure change needed for *E. coli* cells to enter 700 nm tall constrictions with varying widths. As the constriction width reaches 1.5 µm, the force needed to move cells into the constriction increases significantly. Error bars indicate standard deviation. 127

Figure 43: Applied pressure change needed for *P. aeruginosa* cells to enter 950 nm tall constrictions with varying widths. 128

Figure 44: Applied pressure change needed for *P. aeruginosa* cells to enter 700 nm tall constrictions with varying widths. * indicates P < 0.05 from ANOVA test. # indicates sample size is 2 for that constriction. 129

Figure 45: Images of *E. coli* and *P. aeruginosa* passing through the constrictions. (A) Brightfield and (B) RFP filtered images of *E. coli* entering 950 nm tall, 1.5 µm wide constriction at 600 mbar of applied pressure. (C) Brightfield and (D) GFP filtered images of *P. aeruginosa* passing through 700 nm tall, 1.5 µm wide constriction at 10 mbar of applied pressure. 131

Figure 46: (A-C) Brightfield images of *S. aureus* at a 700 nm tall, 2 µm wide constriction entrance after varying applied pressure. Though the applied pressure was increased, the cells could not enter the constriction and accumulated at the entrance of the constriction. (A) 50 mbar. (B) 75 mbar. (C) 100 mbar. Scale bar: 50 µm. 132

Figure 47: (A-C) Brightfield images of *Psychroserpens* sp. at an 870 nm tall, 1.15 µm wide constriction entrance after different applied pressure values. The constriction was narrow enough to prevent cells from entering, resulting in accumulation of cells at the entrance of the constriction. (A) 100 mbar. (B) 300 mbar. (C) 500 mbar. Scale bar: 50 µm. 132

Figure 48: (A-B) 40X and 100X magnified brightfield images of one part of sub-microfluidic device, showing the morphological changes in *S. aureus* as the cells were exposed to penicillin/streptomycin, respectively. The cells became swollen upon contact with the antibiotic. 134

Figure 49: (Top) SPRi difference image 21 hours after the start of the experiment. Lower brightness intensity indicates less biomass accumulation. (Bottom A, B) Brightfield optical images of the regions shown at the top image. In these images, a darker color represents a greater biomass density (adapted from [280]). 135

Figure 50: (A-B) Optical microscope images of bonded PDMS-gold surface devices with 950 nm tall constrictions. The gold surface was functionalized with 25mM MPTMS for 3 hours. (A) 1.5 µm wide functional constriction. (B) 2.5 µm wide partially collapsed constriction. 136
Figure 51: (A-B) New platforms to enable the isolation of both motile and non-motile species from the environment. (A) A glass device (B) A high throughput Si-wafer that could contain 384 devices............ 139
LIST OF TABLES

Table 1: Separation efficiency data of devices: 49 constrictions with various widths were used to separate a culture of CFP P. aeruginosa and m-cherry E. coli mixed at a same v/v and concentration ratio (adapted from ref [250]). ................................................................. 93

Table 2: A comparison between the frequency of taxonomic level obtained with the devices to the conventional plates. .................................................. 114

Table 3: Species richness in devices and plates with respect to number of isolates collected. ....................................................................................... 114

Table 4: Number of species grown in each isolation chamber of sub-microfluidic devices with respect to the width of 700 nm tall constrictions .......................................................................................... 116

Table 5: Number of species grown in each isolation chamber of sub-microfluidic devices with respect to the width of 950 nm tall constrictions .......................................................................................... 117
1.0 Introduction

The majority of the microbial world is still mostly unexplored, much less understood. Identifying and studying these new species can address the underlying factors of many diseases, result in the discovery of new drugs, and reveal the missing pieces of the microbial world [1]. Collecting samples from the environment does not yield significant information due to the constraints of isolating and cultivating these species [2, 3]. The main issue is that most organisms do not grow in standard laboratory growth media, requiring certain chemical cues and conditions to proliferate. A breakthrough in genetic sequencing in the 1980s has led to significant amounts of new information about microbial communities and diversity. 16S rRNA analysis and recent publications on the human microbiome project reveal that there are thousands of unstudied bacterial species in the environment [2, 4-7]. Furthermore, the genetic analysis of infected samples almost always indicates the presence of multiple species though the commercial media used for the detection of bacterial infections often culture only one or two well-known species [8]. Metagenomics reveals some information regarding the function of genes or the molecules produced by them, but isolation and cultivation of bacteria is necessary for scaling up and doing further analysis on these species. That is why studies have now started to focus more on isolating and culturing this unstudied majority.

Several groups focused on determining the right compounds or the right growth conditions to grow these cells [9-11]. Short amino acid sequences, cyclic amp, and homoserine lactone are only a few of these molecules found to induce the
growth of uncultivable species [12, 13]. While these techniques certainly improve the growth of several species, obtaining a high throughput is very challenging.

Research groups now focus more on miniaturized systems to tackle this problem. Kaeberlein et al. developed a diffusion chamber, which was later improved by Nichols et al. to iChips [14, 15]. These systems enable the exchange of nutrients from natural environments into the chambers via nanoporous membranes to grow uncultivable species. Researchers also reported that it is significantly easier to maintain cultures in the laboratory once pure cultures of novel species are isolated, a phenomenon known as domestication [3, 14].

Microfluidics is an excellent tool to perform various studies, including Polymerase Chain Reaction (PCR), proteomics, chemical synthesis, and droplet-based techniques by adding micro-valves, pumps and mixers into these platforms [16, 17]. Due to the small size scale that microfluidic systems provide, it is possible for researchers to monitor how bacteria move and behave in microenvironments that are very similar to nature. Methods including, dielectrophoresis, droplet-based cell sorting and fluorescent activated cell sorting (FACS) have been implemented to capture and sort cells [18-23]; however, the complexity and the cost of these instruments prevent their extensive use. Micro-scale confinements to capture and sort bacteria provide advantages in the analysis of bacterial cells with the promise of high-throughput and low-cost production. They also elucidate the behavior of bacteria more realistically than normal culturing techniques since bacteria exist mostly in micro-porous environments such as soil and decomposing matter [24-27]. The main drawback of these studies is that they do not allow the isolation of the
captured cells or yield pure cultures. Furthermore, these designs are applicable for *in vitro* studies rather than *in situ* experiments.

This thesis proposes a novel approach that applies size-dependent sorting to isolate bacterial species in heterogeneous samples, including environmental and patient samples. The size and shape of bacteria differ significantly with diameters ranging from sub-micrometer to tens of micrometers [28]. This lab-on-a-chip design could also be optimized to study bacterial behavior in confinements under applied pressure, which is critical for the separation and filtration techniques such as slow sand filtration method, which is used for obtaining potable water. Polydimethylsiloxane (PDMS) polymer microfluidic device containing nano- and sub-micron constrictions with different geometries are developed. These constrictions provide at least one dimension smaller than the diameter of a bacterial cell. As bacterial cells enter the main entrance, fresh food in the isolation chambers create a gradient along the device and chemotactically attract microorganisms toward the constrictions. Bacterial species get trapped at the entrance of these sub-micron constrictions, preventing other bacterial cells from reaching the isolation chamber. The trapped microorganism continues to divide and each progeny advances further through the constriction. After several successions, only one species will enter the isolation chamber, which is the predecessor of the trapped species (Figure 1). These devices are so versatile that bacterial species can also be induced to form biofilm, and studied by forming a chemotactic gradient, or forced into these constrictions under pressure to mimic filtration systems. This thesis proposal includes a detailed critical literature review of the techniques for single bacterial cell isolation and
cultivation techniques, experimental methodology of device design and testing, and results and discussion of each aim.

Figure 1: Schematic of the microfluidic device. Main entrance is connected to isolation chambers via nano- and sub-micron constrictions. Heterogeneous bacterial culture that enters the main entrance self-sort into different isolation chambers with the help of chemotaxis and size-specific constrictions.
2.0 Critical Literature Review

2.1. Single Cell Studies

Most studies to date obtain an average result from a population that contains millions of cells and often show a significant variability in response to a stimulus. This variation is observed as the population behavior masks the actual response of an individual cell [29]. Single cell studies therefore become an important focal point for researchers since it could reveal more information about diseases and cellular interactions. Studies on single mammalian cells started earlier than bacterial cells because bacterial cells are more challenging to capture due to their fast swimming and smaller sizes. These characteristics could also make it more difficult to visualize these cells using simpler microscopes. Nonetheless more studies have started to focus on capturing individual bacterial cells as several methods have been developed. These techniques and their applications to microorganisms will be discussed in detail in the following sections.

2.1.1. Micro- to Nano-Scale Systems

2.1.1.1. Single Cell Isolation

Microfluidics has become a unique and an essential tool in biological applications as nanotechnology significantly advanced, and the research projects became more and more interdisciplinary that the semiconductor technology was integrated into life sciences research. Microfluidics is defined as a fluidic system that has dimensions of 1 micrometer to hundreds of micrometers, and contains volumes on the order of nanoliter to femtoliter [16]. There are several advantages of
microfluidics that makes the technology very attractive. One could start working
with only a small number of cells which could be conveniently observed under a
microscope over time, analyzed and manipulated. It requires only small amounts of
reagents, and samples for reactions, and provides high-throughput screening [17].
The chips could be constructed from simple designs to very complex
microelectromechanical systems that include valves, pumps and mixers to create lab-
on-a-chip reactors, separators and many more applications [30-32].

Micro- to sub-microchannels with different geometries were used for
studying individual bacterial cells since these channels are excellent in cell capture
due to their size scale. Some of which have taken advantage of fluid flow in
microfluidic systems. As an example, Li et al. fabricated 15 µm deep U-shaped
structures to trap yeast cells in [33]. Di Carlo et al. proposed a similar, yet a better
approach in terms of design and micro fabrication. Using cup-shaped micro traps
and hydrodynamic forces they were able to trap individual mammalian cells into
designated sites [34]. Kim et al. devised a remarkable hydrodynamic trapping
method. Micro sieves with a width of 0.8 µm were constructed to immobilize cells as
the fluid flowed through the channel. This approach provides continuous monitoring
of single cells, but cannot be performed at high flow rates or at low cell numbers
[35]. Liu et al. employed a similar structural design with optofluidic imaging to
detect waterborne pathogens from their morphology and refractive indices [36]. An
optofluidic immersion refractometry was integrated to an array of 50 U-shaped traps
with 500 nm gaps to detect the refractive indices of bacteria from the optical contrast
between the cells and the medium. The group was able to distinguish three common
waterborne pathogens, *Escherichia coli* (*E. coli*), *Shigella flexneri*, and *Vibrio cholera* from differences in size, shape, and refractive index value. Probst et al. immobilized *E. coli* cells in sub-micron traps while constantly flowing fresh media to replace any waste products and remove daughter cells. With this technique, they observed the growth kinetics of individual cells for several hours under a time-lapse microscope [37]. Interestingly, Huebner et al. designed a device with an array of traps to capture, incubate and recover individual droplets that contain *E. coli* cells with hydrodynamic forces, instead of directly trapping bacterial cells. This technique is aiming to monitor enzyme kinetics in separate compartments as well as bacterial growth rates [38]. Guo et al. introduced a distinct approach to achieve size-specific separation of cyanobacteria from *Chlamydomonas*. They track-etched conical nanopores in polyethylene terephthalate, and placed the polymer in between two PDMS layers. The top and bottom pore sizes were kept approximately 1.3 µm and 270 nm, respectively. The authors captured and released cyanobacteria in and out of the pores by applying pressure in different directions [39].

Another approach is to cross-link proteins to introduce distinct microstructure. Connell et al. captured a single bacterial cell and investigate quorum sensing by creating The 3D "lobster traps", which are made of Bovine Serum Albumin (BSA) protein, and can enclose picoliter volumes within. Each structure traps bacteria by utilizing the conformational change of the protein at different temperatures. The main limitation of this method is that it can only be applied at laboratory conditions and not *in situ* [27]. In their other paper, the group used laser lithography to create 3D compartments surrounding the selected bacterial cells,
trapping them and allowing small bacterial communities to be studied under a microscope. For this purpose, they used a solution containing gelatin and photosensitive molecules to stimulate cross-linking of the polypeptide molecules. 3D laser printing enables the construction of various designs so that different species can be encapsulated separately, while they can still communicate with each other through pores. The group showed that *Staphylococcus aureus* (*S. aureus*), which is known to be susceptible to beta-lactam antibiotics, could gain resistance to the antibiotic when enclosed with *Pseudomonas aeruginosa* (*P. aeruginosa*) in these structures [40]. Kaehr *et al.* cross-linked proteins in distinct 3D designs by using multiphoton lithography to track bacterial movement. The results showed that the bacterial cells can be directed into certain pathways with the help of specific wall trajectories, confinement, and fluid flow [41].

There are other microfluidic designs that do not rely on hydrodynamic forces to trap bacteria. Nam *et al.* created constrictions with varying heights by using multilayer photolithography to separate bacterial cells they obtained from the Lake Michigan based on size differences [42]. This set-up; however, contains only a couple of constrictions, and requires fluid flow to separate cells. Unlike this method, our approach is completely passive, and can be placed *in situ*, allowing environmental species to be isolated, cultivated and collected. Han *et al.* self-sorted *Caenorhabditis elegans* with respect to their stage of growth, which is comparable to their sizes [43]. To achieve this, they built a microfluidic device with an array of microchannels specific to the moving pattern of the worms at each growth stage. An external electric field was applied to enhance the directional movement of the
worms, facilitating the self-sorting. This example highlights how microfluidic devices with micro- to nanoscale constrictions can be used to separate organisms with respect to their sizes.

### 2.1.1.2. Bacterial Cell Cultivation

Many groups have used microscale channels as “microchemostats.” Microchemostats are microscale bioreactors that allow controlled growth of bacteria by continuously feeding the system with fresh nutrients and removing the waste products. These miniaturized cell culture reactors provide an excellent environment to study the growth behavior of bacteria, as cells can reach a predetermined concentration much faster in these systems than in traditional macroscale chemostats. Monitoring bacteria at single cell resolution and easy handling of the system are among the major advantages. The design and fabrication process of microbioreactors could be found in detail in extensive review papers [44, 45].

Understanding bacterial growth characteristics in real-world situations is a critical research topic for microbiologists. A precise knowledge of the growth kinetics, conditions that affect the growth, and the growth trends of cells as they interact with other species are needed for optimization of large scale bioreactors. Until recently, these studies were completed mainly by using bench scale setting to grow bacterial cultures and the results extrapolated. Large-scale systems use significant resources and become labor intensive, thus limiting the number of variables that can be tested. In their review paper, Brehm-Stecher et al. explain the discrepancies between single cell and population level studies [46]. Microfluidic
chemostats can provide information using only a few cells or even single cells and confirm the differences between size scales.

One of the first examples of a fully-functional microchemostat-on-a-chip was shown by Balagadde et al., who observed *E. coli* growth kinetics in six parallel and independent chemostats fabricated in a single chip that were capable of removing biofilms from the microreactors [47]. Gan et al. employed a similar approach and concluded that the final cell concentration reached in microfluidic devices is comparable to the conventional cell culture concentrations [48]. Cell growth in microfluidic devices was enhanced by adding micropumps to increase oxygen concentrations in the culture medium. The group observed that at the end of 6 hours, the final cell concentration of *E. coli* in the microchemostat with a micropump system reached $2.9 \times 10^9$ cells per mL, which is similar to cell concentrations measured during the stationary phase of large-scale cultures. Ingham et al. built a million micro-well growth chip on a porous aluminum oxide material and placed it on top of an agar plate to culture slow growing bacteria in separate compartments without bias, and conducted high throughput screening [49].

Several design parameters have been added in microchemostat to test growth conditions, to observe morphological changes, or to run multiple experiments in parallel [50-52]. Zanzotto et al. measured pH, dissolved oxygen, and biomass in terms of optical density (OD) in microbioreactors with sensors, and compared their results with the results obtained with 500 mL-bench scale reactors. Microbioreactors provided very similar results in terms of growth parameters and bacterial behavior compared to the larger scale studies [53]. The same group also conducted gene
expression studies with *E. coli* in both nutrient rich and minimal media, and determined the changes by using only 500 ng of RNA from *E. coli* [54]. In another example, Grunberger *et al.* cultured industrially relevant bacterial strains, such as L-lysine producing *Corynebacterium glutamicum*, in picoliter volume to analyze the morphological changes in bacteria under different conditions. Importantly, their study highlights that a microbioreactor with single-cell resolution provides more detailed information of bacteria in a very short amount of time, which would not be possible with high density cultures [55, 56]. Steinhaus *et al.* applied microbioreactors to investigate the growth conditions of methanogens, which need anaerobic conditions to grow [57]. Additionally, microchemostats is a great tool to study interactions between bacterial species such as prey-predator relations [58].

Instead of confining bacterial populations into a microreactor, many researchers have taken a different approach and designed microchannels in order to observe bacterial growth at a single cell level. Moffitt *et al.* made microfluidic devices from agarose, instead of using conventional PDMS polymer [59]. The authors reported that agarose is more elastic and flexible, which facilitates the growth of several distinct species with different morphologies, and enables a more effective nutrient transport than PDMS does. The designed agarose tracks were narrow enough to confine cells but flexible enough to allow cells to retain their natural shapes so that *E. coli*, *Bacillus subtilis* (*B. subtilis*), and *Enterococcus faecalis* could separately grow in the same size tracks. The group also could grow auxotrophs in agarose tracks when these cells were cultured with other species. Inoue *et al.* examined the growth rate and the cell size of both individual and a group
of *E. coli* cells by simultaneously imaging them with phase contrast/fluorescence microscopy in microchambers. The chips were constructed by etching thick glass slides, and sealed with semi-permeable membranes by using biotin-streptavidin attachment [60].

Wang *et al.* designed a PDMS microfluidic chemostat, called the “mother machine,” to trap a single *E. coli* cell, and study its growth kinetics for several generations. These devices included an array of growth channels that have dimensions of 25 μm in length x 1.5 μm in width x 1.4 μm in height and were connected to one large trench that continuously fed fresh food to the cells. With the help of this design they concluded that the death of *E. coli* is not caused by random events like DNA damage but the accumulation of lethal factors [25]. Moolman *et al.* changed the fabrication technique to construct narrower sub-micron lines by using electron-beam lithography, but maintained a similar design. They monitored the growth of *E. coli* and *Lactococcus lactis* for generations [61].

According to Long *et al.*, one disadvantage of Wang’s design is that only one side of the growth channel is open to a fresh food source and it is the only exit point of bacteria inside the growth channels. Therefore, the bacteria in the growth channels are at different ages and filamentation (cell extension without division) is observed due to accumulation of damage [26]. To prevent these problems from occurring, they modified the design so that growth channels are connected to large trenches on both sides [62]. Two open ends enable cells to overflow from both sides, maintaining a constant bacterial age inside the channel. They varied the width of the growth channels from 0.6 μm to 0.9 μm while keeping the height 1.1 μm to obtain the ideal
geometry for trapping bacteria in 1-dimension. Bacteria were trapped in the growth channels by creating a pressure difference between the two large trenches. When the pressure difference was eliminated, the channels were narrow enough to prevent bacterial movement, allowing high-resolution imaging to monitor bacterial growth [26]. Balaban et al. studied bacterial persistence in *E. coli* by enclosing them in narrow channels that were sealed with membranes. They exposed the cells to ampicillin for few hours until they stopped growing, and then flushed the device with fresh growth medium to remove the antibiotic. They observed some bacterial cells, known as persisters, start to grow again in the fresh growth media. They concluded that these cells have a slower growth rate than normal cells, indicating the heterogeneity in bacterial population [63]. In another example to such studies, the effect of isoniazid, a bactericidal antibiotic, on the persistence of *Mycobacterium smegmatis* cells was investigated [64].

One focal point in microchemostat research is to achieve long-term culturing while continuously and precisely controlling the growth conditions such as temperature and cell density. Au et al. set up a digital microfluidics device that could control a droplet of bacterial culture for five days with the electrodes located inside the device to mix the growth medium and regulate temperature and cell density profiles [65]. There are other studies that also tried to improve the growth conditions for continuous cell culturing in microchemostats [66]. Johnson-Chavarria et al. fabricated an automated single cell microbioreactor to grow bacteria. Their set-up included syringe pumps to control the flow rates, a pressure control system to regulate the valve pressures, and a microscope to observe the cell trapping.
Temperature in the system was controlled by thermocouples. By using their single cell microbioreactor (SCM), they were able to trap single *E. coli* cells to study the doubling times and changes in gene expression in different media [67]. Another study showed how bacterial populations adapt and evolve under different conditions by fabricating micro habitat patches (MHPs), which were connected to other MHPs and to feed lines by nanoslits [68].

2.1.2. Droplet-Based Cell Capturing and Cultivation Methods

Droplet-based platforms in microfluidics have diverse biological applications from miniaturization of polymerase chain reaction (PCR) [69-72] to single cell detection and analysis [73, 74]. Micro-scale droplets are generally created using two immiscible fluids (mostly oil and aqueous phases) in a continuous fluid flow system and have volumes ranging from femto- to nano-liter [73, 75, 76]. One main advantage of this method is that it allows many experiments to be carried out at the same time in independent compartments [72, 77]. Droplets provide a unique platform for cellular experiments, enabling detection and observation of single cell behavior while it can still communicate with other cells by exchanging important factors and chemicals through the immiscible fluid [18]. A single cell within a droplet could be obtained by diluting bacterial cultures to a cell density that will lead to 1 cell per droplet volume. There are also studies that have focused on other strategies for obtaining individual cells. Park *et al.* constructed micropillar arrays and applied hydrodynamic forces to improve the separation of bacterial clusters in various cell concentrations before encapsulating single cells into hydrogel droplets.
These platforms can be designed as an automated droplet technique to culture bacterial cells for longer durations by manipulating the droplet content [79].

Droplets have been extensively applied for mammalian cell studies. Many different cell types can be encapsulated into these droplets and examined simultaneously [75]. Although bacterial cells are more challenging to capture in droplets due to their small size, fast motility and growth rate several research groups have started focusing on examining single bacterial cells in droplets due to the advantages this technique offers.

The applications of microdroplets include the investigation of the antibiotic sensitivity of bacteria [19, 80], the symbiotic relationship of co-cultured microorganisms [81], the quorum sensing behavior of bacteria [20, 21], the enzymatic activity or secretion of secondary metabolites [77], and the cultivation of uncultivable bacteria [18]. Shim et al. trapped individual E. coli cells that can express red fluorescent protein along with the enzyme alkaline phosphatase inside of micro-droplets by using a PDMS microfluidic platform and measured the enzymatic activity by the level of fluorescence protein expression [82]. Rane et al. reported that single E. coli cells can be analyzed by encapsulation microdroplets. The cells were mixed with 16S rRNA complementary probes and after encapsulation the cells were lysed at high temperature and probes were hybridized with 16S rRNA found in the cells [83]. In another example, Park et al. co-cultivated symbiotic bacterial tryptophan and tyrosine auxotroph E. coli, which could only grow in the presence of each other, in minimal media. They rapidly generated a collection of droplets
containing different ratios of the two strains. As little as 1% of one strain was needed for both species to grow in the droplet [81].

Fitzsimons et al. combined a droplet-based single cell capture method, flow cytometry and sequencing to explore the microbial diversity, specifically *Streptococcus* and *Enterococcus* species, in human oral and stool samples. They produced gel microdroplets (GMDs) by mixing immiscible oil with aqueous liquid and melted agar to enclose single bacterial cell obtained from human oral and stool samples, incubated them for one day, sorted them by using flow cytometry and applied multiple displacement amplification (MDA) to amplify the genetic codes collected from each GMD. This method allowed the cultivation in the droplets from a single cell, which increased the number of identical DNA sequence copies and resulted in more accurate sequencing data [22, 84]. Another GMD study was performed to enrich *Leuconostoc mesenteroides* from *Bacillus subtilis*. *L. mesenteroides* cells in GMDs were distinguished from *B. subtilis* cells due to their inability to form microcolonies, and sorted with flow cytometry after being stained with fluorescent dye [85]. A similar method was implemented to sort slow-growing marine bacteria from the fast-growing ones using Fluorescence Activated Cell Sorter (FACS). After staining the cells with fluorescent nucleic acid stains, 12 marine microorganisms were individually encapsulated in gel microdroplets. As the bacteria grew, fluorescence intensity in each droplet increased and varied as the species have different growth rates [86].

Similar to droplets, Martin et al. used immiscible liquids to build a segmented fluid system in Teflon tubing, with volumes ranging from 4 nL to 100 nL
and studied four different microorganisms. Like other miniaturized culture techniques, the growth rates of microorganisms, including the strictly aerobic *Pseudomonas fluorescens* were in good correlation with the growth rates reported in large culture volumes [87]. The Ismagilov Lab created stochastically occupied confinements by using plug-based microfluidic devices. They isolated individual cells from heterogeneous samples into separate plugs and cultivated bacterial cells, and then split the plugs to encapsulate one daughter cell into each plug. The group carried out various analyses, including cellulose assay, Gram-staining, identification of cells by Fluorescence *In Situ* Hybridization (FISH) assays, using this versatile approach [88].

Another versatile droplet-based microfluidic system was proposed by Leung *et al*. They fabricated a programmable chip that can sort and isolate bacterial species, culture from a single cell, and conducted quantitative PCR and whole genome amplification for genomic analysis of individual species. One major difference of this system is that the immobilization of droplets was achieved by flow-control wetting, rather than surface tension and hydrodynamic trapping [89].

Droplet-based cell capturing is often integrated with highly sensitive detection techniques such as fluorescence probing, Raman, or mass spectroscopy to rapidly collect data from individual droplets [73]. FACS is often coupled with droplets to detect and sort bacteria in various samples ranging from the environment to sick patients [90-93]. Eun *et al.*, for instance, encapsulated *E. coli* in agarose microdroplets and utilized FACS to determine antibiotic sensitivity levels and to detect resistant mutants [80]. Although flow cytometry has high sensitivity, this
instrument is very expensive, requires trained personnel and needs fluorescence tagging of specimens for detection and sorting [94, 95]. More information regarding these systems can be found in review papers dedicated to droplet-based studies [73, 74].

2.1.3. Electrophoretic Separations, Optical Tweezers and Other Techniques

In addition to microdroplets and microscale confinements to capture and sort single bacterial cells, researchers have proposed several other techniques, including electrophoretic separation, including dielectrophoresis and capillary electrophoresis, optical tweezers, and so forth. In the following sections, these techniques will be discussed in detail, and the examples for each method will mainly focus on the advances in bacterial separation.

2.1.3.1. Electrophoretic Separation Techniques

The principle of electrophoretic separation of particles is the movement of particles under an applied electric field generated by direct or alternating current. Dielectrophoresis (DEP) and capillary electrophoresis (CE) are both extensively used in the literature to separate biological particles. In 1951, the pioneer of dielectrophoresis (DEP), Pohl, described this method as the application of non-uniform electric fields on particles. Though it shares a very similar principle with electrophoresis, particles do not need to have a charge for separation with dielectrophoresis. Instead, a non-uniform alternating current (AC) creates a dipole on particles and induces separation [96, 97]. The conductivity of the solution and characteristics of the applied field affect bacterial separation [97-99]. Electric fields are often generated by microelectrodes, which can be easily fabricated by following
the same photolithography steps that microfluidic devices are fabricated with. Microelectrodes achieve the necessary DEP force for separation by applying lower voltage than regular electrodes do, resulting in less heating and fewer electrochemical effects [100]. Studies also showed that both Gram-positive and Gram-negative bacteria have a negative charge on their surface, but the magnitude of the charge varies with strain [101, 102]. Markx et al. conducted a similar study to optimize DEP for the separation of bacterial species by changing various conditions [97, 103]. They investigated the effect of conductivity and fluid flow rate on the separation of Bacillus subtilis, E. coli, and Micrococcus luteus and determined that the conductivity of Gram-negative bacteria (E. coli in this study) is very different than the conductivity of Gram-positive bacteria, and that they can escape from a DEP chamber at lower conductivities [97].

Different modifications were done to increase the efficiency of bacterial separation. DEP was implemented into a microfluidic device to sort rare cells, which were tagged with marker proteins on their surface. Hu et al. observed that 99% of E. coli cells which expressed special marker proteins on their membranes were separated out from normal E. coli species [23]. This method can be easily applied to differentiate mutated cells from healthy cells, but the question arising is how many cells have specific marker proteins that can change the charge on the bacterial membrane. Other studies demonstrated that live and dead bacterial cells could be separated at high efficiency [104, 105]. Lapizco-Encinas et al. developed an insulator-based (electrodeless) dielectrophoresis set-up instead of microelectrodes to segregate live and dead E. coli cells [105]. Furthermore, Donato et al. investigated
the effects of negative and positive DEP on bacterial viability and metabolism as the bacteria were trapped in a microfluidic device. For this purpose, the authors used GFP labeled *E. coli* cells that expresses the fluorescent protein in the presence of an inducer to detect viability, and they applied electric field using electrodes in a quadrupole configuration. The study demonstrated that under both negative and positive DEP, *E. coli* cells were concentrated without having any damage though they observed loss of viability when the exposure to electric fields was prolonged [106].

Fritzsch *et al.* introduced a novel negative DEP method that effectively isolates both Gram-negative and Gram-positive bacteria that are motile and non-motile, and provides contactless cultivation, which could prevent phenotypic changes occurring in bacteria [107]. “Envirostat 2.0” is a miniaturized device that contains an optimized microelectrode and microchannel system. The electrode design, which is in an octupole arrangement, is miniaturized down to 20-12 µm for the isolation of 5-16 µm and 0.5-8 µm size cells, respectively. The successful miniaturization of the microelectrodes decreases Joule heating and avoids damaging the cells, which is an important aspect of this method. Dusny *et al.* applied this microfluidic system to trap individual cells and monitor contactless bacterial growth under controlled conditions. They observed that controlled environments resulted in very high growth rates in microorganisms and argued that the negative effects of DEP on bacterial cells were avoided by choosing the lowest voltage and highest frequency settings [108].
Cai et al. illustrated an application of DEP based bacterial separation technique in clinical samples [109]. They diluted whole blood samples and loaded them into a SlipChip device, which was first introduced by Du et al. in 2009 [110]. SlipChip is composed of two patterned pieces that can be slipped past each other to introduce new chemicals or processing steps. For example, one stage can fill a microwell, the next stage will connect the ducts to the wells for establishing diffusion gradient in the system, and the finally stage will allow the bacterial samples to be collected from the microwell for further analysis. One potential difficulty in the system is leakage, which is prevented by fluorinated oil. It also helps reduce friction for slipping between stages. Cai et al. combined the device with a DEP platform to separate bacterial cells, and with an array to conduct PCR with end-point fluorescence to identify species. The authors were able to identify common pathogens such as *P. aeruginosa*, *S. aureus*, and *E. coli* in three hours [109]. Braff et al. were able to separate different strains of *P. aeruginosa* using a dielectrophoresis device [111].

Capillary electrophoresis (CE) is another electrophoretic approach that is carried out by applying a direct current (DC) across a capillary. CE has also been used to sort bacterial species [112-114]. This technique is advantageous to segregate charged particles such as proteins, DNA and antibodies; however, it is challenging to observe the same success with bacterial species. Finally, modifications in electrophoretic methods have been done to improve these techniques and increase the yield [115, 116]. As an example to these modifications, Huang et al. incorporated AC and DC currents to overcome the limitations of EP and DEP and
manipulated both charged particles (antibodies) and microsized particles (bacteria) [115].

These techniques have different approaches for separating and detecting different species; however, it is not possible to use the devices directly in the nature such as placing them into lake or ocean water. Our proposed approach provides a simple, passive, yet a very novel and effective way to isolate species in situ.

2.1.3.2. Optical Tweezers

Optical tweezers are one of the many techniques used to study single cells [117]. Optical tweezers mainly use gradient forces exerted by the laser beam light, which produce variable electric dipoles to manipulate species. By using a high numerical aperture microscope objective lens the light can be focused to a focal point and its intensity can be used to stably trap particles. [118-120]. A continuous-wave (CW) diode laser is an extensively used light source for trapping cells [121]. The major issue with this method is heating. The heat created from light can damage the cells; hence substantial optimizations must be done to avoid this problem [122]. Ashkin et al. indicated this issue on their paper and stated that the use of infrared-laser light instead of argon-laser light limited the heat damage in bacteria. They demonstrated the proliferation of E. coli within infrared traps [123]. Neuman et al. showed that long term near-infrared laser trapping damages cells, specifically photo damaging at aerobic regions [121].

Enger et al. coupled this technology with microfluidics. They moved bacteria into reservoirs and examined the cells as they were exposed to different media, and sorted them one by one into different channels using optical tweezers. This system
was also used to select and isolate a single cell from a population of cells [124]. Min et al. expanded this idea by using optical tweezers to hold *E. coli* cells in place and monitor the motion of their flagellum. By monitoring the cells over time, the authors were able to see the frequency at which the *E. coli* used its flagella to move in a defined direction vs. tumbling. Understanding the frequency of flagella rotation as cells get exposed to different chemo-effectors will play an important role in predicting bacterial response *in vivo* [125]. Probst et al. isolated and manipulated single *E. coli* cells to monitor their morphologies in a microfluidic device. The authors claimed that for the first time a filamentous *E. coli* cell was separated from its colony, and noted that the method did not damage the cells since the *E. coli* cells continued to divide [126].

2.1.3.3. Other Techniques

In addition to the techniques mentioned above, there are many other technologies that have either been integrated with microfluidic devices or used as standalone methods for single cell capture and analysis such as immunomagnetic separation, magnetic nanoparticle, and quantum dot-based immunoassays [127, 128]. In one such example, Ye et al. employed magnetic particles to induce rotational flow and accomplish the movement of motile *Serratia marcescens* on a surface in 2D, instead of using optical tweezers, which cause heating and damage to bacterial cells [129]. Mujika et al. added a magnetic biosensor system into a microfluidic chip and observed *E. coli* cells by creating an antibody-antigen-antibody sandwich [130]. Initially, antibodies were immobilized onto the sensor, and then bacteria were flowed through to allow the cells to attach to the antibodies. Special antibody-
conjugated paramagnetic microspheres were then attached to bacteria. Exposure to the magnetic field created a response on the magnetic sensor, which led to detection of pathogenic *E. coli* cells. Another study proposed selective bacterial sorting by integrating micromagnets into a microfluidic device. The idea is to sort magnetically labeled cells by applying a magnetic field while the unlabeled cells swim away without being captured by the micromagnets [131].

### 2.2. Bacteria

Bacteria are very diverse group of organisms with millions of species on Earth [132]. They exist everywhere from oceans to soil, glaciers to thermal vents, and inside the human body. It is estimated that there are ten times more bacterial cells in human body than human cells [133, 134]. Bacteria play a significant role in chemical cycles such as nitrogen fixation and biodegradation, produce antibiotics and many commercially important molecules, but also cause detrimental disease [135-138]. In the following sub-sections, microorganisms, and their behavior including chemotaxis, antibiotic susceptibility, and movement will be discussed in detail.

#### 2.2.1. Gram-Negative and Gram-Positive Bacteria

Microorganisms are classified into two categories depending on their Gram-staining: Gram-positive and Gram-negative. Upon applying crystal violet stain to bacterial cells, both Gram-negative and Gram-positive bacteria become crystal violet while Gram-negative cells lose crystal violet color in the following decolorization step due to their thin peptidoglycan wall. When safranin is applied to both bacteria, Gram-negative bacteria become counter-stained with safranin as Gram-positive cells
maintain their crystal violet color [28]. Gram staining is an excellent tool to easily distinguish microorganisms in terms of their cell wall structure. Gram-negative bacteria have a thin peptidoglycan layer compared to gram-positive bacteria. This difference provides more physical flexibility to Gram-negative bacteria [28]. Mannik et al. suggested that the rigid membrane structure of Gram-positive bacteria reduces their ability to pass through constrictions narrower than their own size [24].

2.2.1.1. Chemotaxis

Chemotaxis is a phenomenon that explains the behavior of bacterial movement in the presence of a chemical gradient. Bacteria continuously sense their environment for chemical cues and move toward chemo-attractants and away from chemo-repellents. This trait is essential for their survival since they need to find food sources to proliferate and avoid toxic environments [28, 139]. It is known that they show chemotactic movement toward certain amino acids [140, 141].

Microfluidic systems are ideal for chemotaxis experiments because their laminar flow characteristics make it easy to develop and monitor chemical gradients. The two most common ways of creating concentration gradients in microfluidics are fluid flow and diffusion. Both methods have their own advantages and drawbacks. Diffusion-based systems create a stable chemo-effector gradient in the system to monitor the movement of bacteria without any disturbance from the fluid flow. However, it might take a long time to create a stable gradient. Flow-based systems provide an excellent setting for understanding bacterial chemotaxis in environments where there is constant fluid flow. In addition, the gradient is established in few
seconds. Additionally, there are hybrid technologies where researchers have combined both systems.

Diffusion-based chemotaxis is conventionally performed with capillary assays and swarm plate tests [142, 143]. One major drawback of these methods is that the chemo-effector gradient changes with time, causing inaccurate results. Swarm plate assays also require effectors that can be metabolized. Microfluidic systems provide laminar fluid flow and high mass transport that help overcome these limitations. Laminar flow occurs in pipes when the Reynolds number (Re) is less than 2000. Due to the small sizes of microfluidic channels, Re is generally less than 100 [144]. Hence, layers of flow do not mix and mass transfer happens only by diffusion, which provides a remarkable environment to create stable gradients and quantitatively study chemotaxis.

Diao et al. fabricated three parallel microchannels from a nitrocellulose membrane with a CO₂ laser. The two side channels contained blank buffer and buffer-chemo-effector while the middle channel was loaded with *E. coli* cells. The authors monitored the chemotactic response of the wild type and mutant *E. coli* to both a chemo-attractant (L-aspartate) and a chemo-repellent (glycerol). The mutant *E. coli* cells, which were immotile and incapable of chemotaxis, did not show any chemotactic response as expected [145]. Cheng et al. modified the microfluidic design developed by Diao et al. by creating channels from agarose gel membrane instead of a nitrocellulose membrane [146]. They claimed that the use of a nitrocellulose membrane brings many challenges that an agarose membrane could overcome, such as the laser fabrication limitations and diffusion of large signaling
molecules through the membrane. They studied chemotactic response of *E. coli* to the attractant α-methyl-DL-aspartate. Furthermore, Ahmed *et al.* tested three different hydrogel fabrication methods to pick the best geometry for the chemotaxis studies. The three designs were different with respect to the positioning of the channels between PDMS and agarose layers. They selected the design which had the test channel and the side channels in PDMS and agarose layer, respectively, and confirmed the effectiveness of the hydrogel-based diffusion devices, both with linear and non-linear gradients by comparing their results with the literature data [147].

According to Choi *et al.*, however, agarose-based membranes also have some drawbacks, such as difficulty in fabricating and controlling membrane thickness, and leakage problems. They proposed a new approach to form hydrogel membranes by crosslinking tetronic-tyramine polymers *in-situ* with enzymatic reactions [148]. The authors stated that the degree of crosslinking of this polymer can be easily controlled and the membrane obtained is more robust. As a proof-of-concept, they used *Salmonella typhimurium* to show that stable chemical gradients can be formed in the system to perform chemotaxis studies. Furthermore, the same group developed a second approach for chemotaxis studies. They integrated *in situ* self-assembled polystyrene particles into a microfluidic device to simultaneously create chemical gradients of multiple chemicals [149]. The assembly of polystyrene beads was arranged by capillary pressure, and the porosity was controlled by the particle diameter. Choi *et al.* tested the chemotactic preference of *S. typhimurium* toward three different chemo-attractants: aspartate, ribose, and galactose, and indicated that the bacterial cells showed more directed movement toward aspartate
and became insensitive to the attractants as their receptors were saturated. This method could simultaneously test the effect of different chemicals in cells, which is critical as cells are exposed to multiple stimuli in the environment. Next, Shen et al. used SlipChip technology to initially examine the chemotactic response of *E. coli* cells to aspartic acid and nitrate sulfate and to separate bacterial species depending on their motility and chemotactic response to chemical gradients [150].

As mentioned earlier in this section, flow-based systems can establish stable chemical gradients very rapidly, and are used in several studies even though the shear stress in fluid flow might disturb the chemotactic movement of bacteria. In 2003, Mao et al. developed a flow-based microfluidic system that formed a concentration gradient via diffusion by flowing different concentrations of chemo-effectors in parallel. They exposed bacterial cells to varying concentration gradients in several independent experiments, and studied the chemotactic response of mutant and wild type *E. coli* to L-leucine, L-aspartate, L-serine, and Ni^{2+}. The chemo-effectector concentration formed in the device varied perpendicular to the flow and along the channel while the concentration at any given point was constant at any given time [151]. Englert et al. modified the device developed by Mao et al. by adding a concentration generator device, a design that has two input channels, where chemicals with different starting concentrations can be loaded. These two channels then branch out to multiple connected channels, which enable diffusive mixing and create a more uniform gradient [152]. They also reported that *E. coli* cells move toward the quorum sensing molecule (QSM) autoinducer-2 and isatin (an oxidized derivative of indole) while the stationary-phase signal indole repelled the bacteria.
Next, Jeong et al. used a very similar flow-based microfluidic platform to Englert et al.’s device to rapidly create a linear concentration gradient to investigate the chemotactic response of *P. aeruginosa* to peptone, trichloroethylene (TCE) and 20 amino acids [154].

Lanning et al. constructed a simple T-shaped channel, where flows approach from opposite ends and merge into one main entrance, and studied bioremediation by examining the transverse movement of *E. coli* toward a carbon source under axial flow [155]. Wang et al. used opposite ends of the T-shaped channel as an inlet and outlet to flow the bacteria, and the perpendicular channel of the T-shaped designed to flow non-aqueous phase liquids (NAPL). They tested chemotactic response of *Pseudomonas putida* (*P. putida*) to toluene and *E. coli* to phenol and concluded that chemotactic *P. putida* cells preferentially accumulated in the NAPL region compared to non-chemotactic *P. putida* cells. *E. coli* showed a better sensitivity to phenol than *P. putida* did to toluene. Also as the fluid flow increased, chemotactic response of bacteria to NAPL was stifled [156].

Unlike most chemotaxis studies that investigate model microorganisms, the Stocker lab was interested in understanding the chemotactic response of marine species to nutrient patches in aquatic systems [157, 158]. The proposed device combines both diffusion-based and flow-based systems by initially generating a gradient in a flowing fluid using a micro-injector then monitoring chemotaxis in a static fluid. Hence, the diffusion of nutrient patches created in this microfluidic system resembles the ocean environment.
2.2.1.2. Quorum Sensing and Biofilms

Bacterial quorum sensing is a pivotal phenomenon that allows bacteria to form biofilms, initiate diseases, resist antibiotics, and control other survival behaviors. It starts as bacterial cells secrete quorum-sensing molecules (QSM) to communicate with each other. When the concentration of these molecules reaches a certain threshold, bacteria start to coordinate their activity.

The relationship between quorum sensing and cell density is critical in understanding when and how bacteria start this behavior. Microfluidic devices are outstanding tools for this purpose since they can be used to trap a single cell or few cells inside a microchamber and observe them with microscopy techniques. As mentioned in the Single Cell Studies Section, Connell et al.’s “lobster traps” is one such example this. They elucidated that QS depended on population size, density, and the flow rate of the surrounding medium. They also showed that as few as 150 bacterial cells that are confined closely together can demonstrate reduced susceptibility to gentamicin [27]. Park et al. created a maze geometry with a large central chamber on a chip to investigate quorum sensing [159]. They initially filled the devices with motile bacterial cells and observed their behavior without any external gradient. Over time, the cells rearranged themselves into nonuniform population densities such that GFP E. coli cells moved toward an enclosed area located in the center of the device. When mutant strains, incapable of chemotactic sensing, were introduced, the accumulation was not observed. Similarly, Vibrio harveyi cells, which are motile marine bacteria, tended to accumulate in enclosed spaces, such as channels with dead ends. Since no other gradients or variations were
present, these experiments showed that the cells secrete molecules attracting other cells to them and indicate that cells prefer to reside in high-density populations. When the chemical composition of the growth media in the devices was analyzed at different time points, the concentration of known chemo-attractant molecules increased over time, supporting their conclusion.

In 2010, Danino et al. investigated the fluctuations in the concentration of a QSM, acyl-homoserine lactone (AHL) secreted by E. coli. A monolayer of E. coli cells was maintained at a constant density in a microfluidic device with rectangular traps by adding and removing cells with fluid flow [160]. The group noted that there is a synchrony in quorum sensing and that quorum sensing response varies with spatial dynamics as they observed the cellular expression of GFP in a group of cells changed with respect to both location and time. Bai et al. designed a double-droplet trapping system on a chip to understand intra-species quorum sensing and detect the threshold concentration of the autoinducer needed to initiate a quorum sensing response [21]. The authors encapsulated a single E. coli cell that was modified to express quorum sensing receptor protein, LasR, in a droplet and placed the autoinducer N-(3-oxododecanoyl)-L-homoserine lactone into the second droplet. As a second experiment, they replaced the autoinducer molecule with a single E. coli cell that secretes the signaling molecules. They monitored the diffusion of the autoinducer and the QS response via the coupled production of GFP protein expressed by the E. coli cells. The study demonstrated that cells located in separate droplets can communicate with each other through the diffusion of signaling molecules and that a 1 µM concentration of this autoinducer triggers quorum
response in *E. coli*. Weitz *et al.* performed a very similar analysis to study the diffusion of inducers, N-(3-oxohexanoyl)-L-homoserine lactone (3OC6HSL) and isopropyl-β-D-thiogalactopyranoside (IPTG), and their induction of quorum response in *E. coli* cells. Their results supported the relationship between spatial distance and effective diffusion of autoinducer molecules to initiate a quorum sensing response in bacterial cells [161]. Boedicker *et al.* confined single cells using a plug-based microfluidic device. These nanoliter ‘stochastic confinements’ concentrate bacterial cells from a diluted sample and improve the detection and response time. The study showed that quorum sensing initiated after 8 hours of incubation with only two *P. aeruginosa* cells enclosed in a 100 fL volume. The group also identified the heterogeneity of the quorum sensing response in *P. aeruginosa* due to variations among individual cells [20]. These results provide a critical understanding that biomass and volume have an important role in initiating a QS response.

Many groups have also incorporated microfluidics into biofilm studies. In 2008, Lee *et al.* focused on how shear stress, and exposure to antibiotics and an enzyme, dispersin B, affects the detachment of *Staphylococcus epidermis* cells from a biofilm by using a simple microfluidic device [162]. The study highlighted that the biofilm morphology and formation changes with shear stress, and that the most effective detachment of the biofilm occurs when dispersin B and the antibiotic, rifampicin, was introduced simultaneously. Next, Kim *et al.* investigated biofilm formation by *E. coli* cells using a flow-cell device similar to the one Englert *et al.* described [152, 163]. This device, however, had 8 chambers that contained a seeding
port to introduce bacterial cells, a diffusive mixer to flow different chemical
gradients and pneumatic valves to control the mixer and the seeding port. Bacterial
cells were exposed to increasing concentrations of 7-hydroxyindole and isatin in
different chambers, and the results suggested that the molecules inhibited and
induced the biofilm formation, respectively.

A different approach to study biofilms is the integration of sensors. Researchers have incorporated multiple sensor types such as electrochemical and
plasmonic sensors to monitor quorum sensing response in bacterial cells, specifically
the pathogenic ones. Webster et al. integrated a sub-micrometer sized
electrochemical sensor into a fluidic system to detect the presence of the redox-
active molecule, pyocyanin, which is secreted by *P. aeruginosa* and can be used as
an indicator of *P. aeruginosa* infection [164]. The group fabricated a microscale
palladium hydride reference electrode and a gold working electrode inside of a
nanoconstriction, detected pyocyanin at a concentration of 0.597 µM by using square
wave and differential pulse voltammetry. Abadian et al. combined microfluidics and
surface plasmon resonance imaging (SPRi) to monitor and detect biofilm formation
and removal in real time without labels [165]. The authors used model
microorganisms, *E. coli* and *P. aeruginosa*, and monitored their movement,
attachment, biofilm formation and removal over time on the surface of a gold coated
prism. One major advantage of this method is that SPRi provides a larger field of
view (1 cm²) compared to optical microscopy, allowing mature biofilms to be
studied [165, 166].
2.2.1.3. Antibiotic Susceptibility

Researchers have focused on designing inexpensive and fast diagnostic tools to examine the mechanism of antibiotic resistance and determine the minimal inhibitory concentration (MIC) needed for the treatment of infections as many bacteria have started to show resistance to antibiotics. Biofilms are also a major cause of decrease of bacterial resistance to antimicrobial agents [167, 168].

Zhang et al. conducted an experiment to observe the development of resistance in a bacterial population. They used a microfluidic device consisting of 1200 interconnected hexagonal wells etched into a silicon wafer and sealed against a flat glass slide. A gradient of antibiotic was created in the device by introducing Lysogeny Broth (LB) from the top of the device and a mixture of LB and ciprofloxacin, an antibiotic, from the bottom of the device. The solutions were pumped through the device at 30 µL/h for 24 hrs. After being inoculated to the center of the hexagonal array, they started moving toward the periphery of the device, where the LB concentration was the highest, and eventually populated the entire device, including the regions with high ciprofloxacin concentrations. The highest cell concentration was observed at the fluid entrance region, named Goldilocks, where fresh food and antibiotic was continuously introduced. Resistant cells were taken from the first device and inoculated under the same conditions into a new device. The images depicted almost immediate growth, and faster population expansion in the device, confirming that the original strain had acquired antibiotic resistance [169].
Cira et al. initially loaded and dried antibiotics inside individual chambers of a microfluidic device, and then introduced bacterial cells by vacuum. As cells were isolated in separate chambers, antibiotics were dissolved into the media, and the MIC levels of antibiotics were identified for multiple bacterial species [170]. Another study screened methicillin-resistant S. aureus against different antibiotics to determine the MIC levels [19].

In 2012, Cao et al. developed a micro-segmented flow technique that can simultaneously carry out thousands of antibiotic susceptibility tests. The device controlled combinations of different antibiotics with syringe pumps and a computer-based flow control system, and tested the susceptibility of E. coli against ampicillin, chloramphenicol, and a mixture of both antibiotics. Additionally, they monitored the interaction of silver nanoparticles with antibiotics and how these interactions affected the viability of E. coli [171]. Later that year, Churski et al. designed a similar automated microdroplet system for determining the antibiotic susceptibility of E. coli cells to combinations of antibiotics. E. coli cells were incubated inside the droplets for 3 hours and their viability was detected with a spectrophotometer as the cells converted resazurin to a fluorescent molecule, resorufin. They initially confirmed the MIC levels of ampicillin, chloramphenicol, and tetracycline for E. coli by comparing their results with the literature data. Then, they reported that the combination of antibiotic pairs could result in complex and distinct interactions with cells such as antagonistic, suppressive, and additive [172].

Choi et al. reduced the time needed for antibiotic susceptibility tests (AST) to rapidly determine MIC levels. Bacteria in agarose were introduced into a PDMS
coated glass device, and capillary valves provided an interface between bacteria in agarose and the antibiotic solution. Agarose immobilized the bacterial cells, so individual cells could be monitored with time-lapse microscopy as they were exposed to the antibiotic solution via diffusion. The MIC levels were obtained with the same accuracy as conventional techniques while decreasing the waiting time from 16 hours to 4 hours [173]. Li et al. took this method a step forward and sandwiched a monolayer of bacteria between a glass slide and an agarose layer. A monolayer of bacterial cells eliminated the complications occurring as monitoring the growth of individual cells [174]. The authors determined the growth kinetics of individual *E. coli* cells while they were exposed to varying concentrations of amoxicillin, and noted the morphological changes in *E. coli* as the antibiotic concentration increased. The MIC and the half-maximal inhibitory concentration (IC50) levels correlated well with the data obtained with standard microtiter plate approach. Kalasnikov et al. took a different approach to reduce the time needed for AST. They created a microfluidic platform to permanently bond *S. aureus* cells onto an epoxy coated glass surface. They applied shear stress or the enzyme, lysostaphin, to damage the bacterial cells and accelerate the cellular response to antibiotics. The stress factor targets the bacterial cell wall, making bacterial cells more sensitive to antibiotics whereas the resistant bacteria do not get affected. The viability of bacteria was tested with a fluorescent dye, Sytox green, which binds to the DNA of dead bacteria. They collected comparable AST data of 16 *S. aureus* strains in as little as 60 minutes [175].
Another important aspect of bacterial research is the removal of biofilm, which make cells significantly less susceptible to antibiotics. Biofilms are layers of bacterial cells in a thick extracellular polymeric substance (EPS) matrix, which protects them from antimicrobial agents, enhance communication and exchange of molecules between cells [176]. These structures are the major causes of chronic diseases, biofouling of the pipes in plants, and deterioration of the exterior parts of ships. Hence the efforts of removal of biofilms are crucial. Similar to antibiotic susceptibility tests, a group of studies implemented these tests to obtain biofilm removal concentration. In one such example, Kim et al. used a flow-based gradient generator to determine the minimal biofilm eradication concentration (MBEC) for fully developed biofilms. They exposed biofilms of \textit{P. aeruginosa} PAO1 to both the minimal inhibitory concentration (MIC) and MBEC of 11 different antibiotics and compared their results with the MIC levels determined for planktonic PAO1 cells. They found that the MBEC levels needed for removing PAO1 biofilms were 8.5-75 folds greater than the MIC levels needed for planktonic cells [177].

\subsection*{2.2.2. Uncultivable Bacteria}

As mentioned in the beginning of this section, bacteria are one of the most diverse groups, and tens of thousands of microbial species are estimated to be unknown. Rappe \textit{et al.} states that the number of bacterial phyla has increased from 12 phyla to 56, and 26 of which are still candidate phyla. This means that not a single species in these 26 phyla has been cultured though their genetic makeup has been sequenced [2, 178]. The key problem is that most bacteria cannot grow in standard media prepared in laboratories. This issue was also realized when the direct
bacterial cell counts were not in correlation with the colony forming units (CFUs), which is a phenomenon known as “great plate count anomaly” [3, 9, 179]. Researchers hypothesized that the high numbers obtained from direct counts might be due to dormant cells or dead cells; however, advances in technology, specifically 16S rRNA sequencing, elucidated the presence of uncultivable species. More detailed information regarding metagenomics and the identification of species will be discussed in the following section. Although these advances show the presence of unstudied microorganisms and collect more information regarding their metabolic activities, bacterial species need to be cultured to gather detailed information about their characteristics and their behavior in the environment [18].

Different growth media and solidifying agents have been experimented by scientists for decades to culture new marine and soil bacteria [180, 181]. Less nutritious media have shown to support the uncultivable species since their natural habitats such as ocean water do not contain high nutrient content, which is known to be toxic to some bacterial species [4, 9, 182]. Several studies have revealed that certain growth factors, bacterial interactions [12, 14], and long incubation times [181, 183] are needed to trigger the proliferation of these species. Among these factors, the presence of homoserine lactone [182, 184, 185], cyclic AMP [185, 186], and short peptide sequences [12] have been indicated to facilitate the recovery of uncultivable bacteria [3]. Also, changes in CO₂ and O₂ concentrations of the cultures can lead to the growth of some uncultivable microorganisms [184]. Janssen et al. were able to stimulate the growth of both known and new organisms by increasing the duration of incubation to 12 weeks, and using 1:100 times diluted growth media.
They noted that the growth of bacteria was improved by changing the solidifying agent from agar to gellan gum, and by adding CaCl$_2$, which accelerates the gel formation of gellan gum [183]. Joseph et al. stated that the modifications in culture media are sufficient to induce the growth of uncultured soil bacteria without developing any complex platforms to mimic environmental conditions [187]. Although chemical composition modifications have improved to discover new species in the environment, the yield rate is very low.

Nichols et al. indicated that some bacteria need certain signaling molecules secreted by other bacteria to grow. They initially observed that Psychrobacter sp. strain MSC33 can grow when it was co-cultured with a “helper” strain MSC105, whose 16S rRNA gene is 99% similar to Cellulophaga lytica. They also identified that 3.5 nM of a 5-amino-acid peptide is necessary to cultivate the uncultivable species Psychrobacter sp. strain MSC33 [12].

Many studies have started to mimic the natural habitat in which microorganisms grow. Kaeberlein et al. designed a diffusion growth chamber that has marine bacteria placed inside chambers enclosed with polycarbonate membranes. These membranes allow the exchange of nutrients from marine sediment into the chambers and prevent the escape of bacteria. They were able to culture uncultivable bacteria in the chambers and use 16S rRNA sequencing identify two new isolates [14]. Bollmann et al. pointed out that diffusion chambers provide a suitable environment for uncultivable bacteria to adapt to laboratory conditions. When the species were transferred between diffusion chambers for a few successions and then cultivated onto agar plates they observed that the species that previously
failed to grow on agar plates could proliferate [3]. Similar to our approach of trapping bacteria, the same group used these diffusion chambers in a reverse manner to trap specifically Actinomycetes, which has as important role in antibiotic production. Instead of mixing agar media with an environmental sample, they used sterile agar and gellan gum media and sealed them with membranes that had different porosities. Polycarbonate membranes with 0.2 μm pore sizes prevented larger bacteria from accessing the media and successfully trapped more Actinobacteria species than conventional techniques can [188]. The role of membranes in culturing bacteria from the environment is significant. Similar to nano-scale microfluidic channels, size-specific pores on the membrane allow nutrients to pass through, but form a barrier to many fast-growing bacteria from reaching the food and outgrowing the fastidious microorganisms [189-191]. The design of the diffusion chamber was improved by Nichols et al. to “iChip”, which contains miniaturized wells that enclose, on average, one bacterial cell per well [15]. The group’s recent paper reported the application of the iChip in soil samples, where they discovered a novel antimicrobial metabolite produced by a soil species [192].

Zengler et al. encapsulated bacterial cells, which were collected from different environments such as seawater from Sargasso Sea and soil from Ghana, into gel microdroplets (GMDs). Growth columns were used to incubate GMDs for a few hours and 16S rRNA gene sequencing performed on the samples identified microorganisms that have not been recorded in any database. The porous structure of GMDs enabled diffusion of molecules in and out of droplets and allowed specimens to receive the necessary growth factors while keeping them apart for proliferation.
Different growth media were flowed through the growth columns and compared. Seawater was found to provide the optimum environment for the uncultivable bacteria from the Sargasso Sea [18].

Another high-throughput culturing technique was developed by Connon et al. to culture uncultivable organisms. Samples were collected from certain locations in Oregon and filtered through membranes. The collected water was used as media and the bacteria on the membrane were used as inoculum. Extinction culturing [193], which uses unaltered environmental water sample instead of natural water used in regular dilution cultures to dilute species, was applied in microtiter dishes, and they were able to culture microorganisms such as SAR11 and SAR92 that have not been cultivated previously in a laboratory. This method improved the duration and the efficiency of cultivation of uncultivable species when compared to the microscopic counts. Nevertheless, they were only able to cultivate species collected in the summer season, and some species have not been cultivated even though their presence was confirmed with the microscopic counts. The authors also suggested to modify culturing media to target new species [9].

Although all these techniques have pointed out the unknown diversity of bacteria in the environment and revealed a small part of it, new methods are needed to improve the discovery and isolation of new microorganisms. Together with recently cultivated species, the total discovered microbial diversity is far from representing the actual bacterial diversity on Earth.
2.3. Metagenomics

Sequencing technology has been advanced significantly that now it is possible to sequence genomes or to identify bacteria from polymicrobial cultures with next generation sequencing. Before these developments, in 1977 Sanger introduced a DNA sequencing technique with chain-terminating inhibitors [194, 195]. In addition to regular deoxynucleotides (dNTPs), Sanger sequencing uses dideoxynucleotides analogs (ddNTPs) as chain terminators. The dideoxy sugar of these analogs lacks a 3’-OH group, which is needed to form phosphodiester bonds with the neighboring nucleotide. Therefore DNA polymerase could not continue the polymerization reaction, terminating the chain elongation. These analogs are also labelled radioactively or fluorescently for detection.

In 1987, Carl Woese pioneered 16S rRNA sequencing to map the bacterial phylogeny and understand bacterial evolution [178]. Prior to this advancement, Stahl et al. used 5S rRNA sequences to analyze the symbionts of two hydrothermal vent animals: worm Riftia pachyptila and clam Calyptogena magnifica [196]. Compared to 5S rRNA, 16S rRNA contains more base pairs, which makes it more challenging to sequence but more reliable for phylogenetic comparisons [197]. Researchers have started to use 16S rRNA sequencing to gather information from uncultivable species without culturing them [2, 4, 5, 178, 197-199]. DeLong et al. showed the presence of Archeabacteria in a coastal marine environment by cloning rDNAs obtained from different coastal areas [200]. Improvements in sequence-based and functional metagenomics, and the recent findings in the human-microbiome project have revealed that there are still thousands of bacterial species present in microbiota that
have never been cultured [6-8, 201-204]. Venter et al. applied whole genome shotgun sequencing to samples obtained from the Sargasso Sea to elucidate the level of diversity in the ocean [205]. Tyson et al. used random-genome sequencing from a biofilm collected from an acid mine drainage in Iron Mountain, California and obtained information about the microbial community in the biofilm and their metabolic functions [206].

Since PCR is a very reliable and well-known technique to amplify genomic information, researchers have different approaches to scale down all the necessary steps into a microfluidic chip [207-209]. Considering the sample size, cost of reagents, handling, improved heat transfer which leads to faster cycling times, and higher throughput, lab-on-a-chip systems are very valuable [210]. Comprehensive analyses of these systems are collected in review papers by Chen et al. and Paegal et al. [211, 212]. Easley et al. showed a complete PCR analysis on a chip, starting with a whole blood sample and finishing with a genetic profile [213]. Similar approaches have been used for analyzing bacteria on a chip [214, 215]. Zhang et al. designed a microfluidic purification device to isolate DNA/RNA from a single bacterial cell and perform quantitative PCR [216].

Unlike other approaches, Rane et al. detected pathogens by using 16S rRNA hybridization in microdroplets and confocal fluorescence spectroscopy without performing any amplification. E. coli cells were mixed with 16S rRNA complementary fluorescent molecule probes, and encapsulated into droplets. The cells were then lysed the probes were hybridized with 16S rRNA sequences, resulting in fluorescence of the probes [83]. Ottesen et al. utilized a multiplexed
microfluidic chip to perform multi-gene analysis on single bacterial cells collected from termite hindgut [217]. The authors designed a multiplexed PDMS chip for the entrapment of individual bacterial cells. Pressure was applied to a PDMS valve layer to trap the cells in 1176 independent 6.25 nL of reaction chambers. Digital PCR experiments run with the cells showed that the genes of interest were only held by 1% of gut microbiota that was studied.

Zeng et al. looked at single cell capture from a different angle. The authors constructed a sophisticated glass/PDMS/glass chip, to generate oil emulsions that were used for trapping single cells and performing PCR [218]. Single cells along with PCR reagents and beads coated with forward primers were trapped together in small droplets. The primers were for genes that were specific to two different types of E. coli being studied. PCR was performed on the droplets. The droplets were then lysed and the beads sorted by multicolor flow cytometry. The beads having one color represented the droplets containing only a single species whereas the beads that were a mixture of the two colors came from droplets containing multiple species. Using a bead-based approach, the authors were able to achieve a detection limit of 1 in 10^5 for pathogenic E. coli O157:H7 cells in a mixture with normal E. coli K12 cells.

The next step for PCR-on-a-chip is to reduce the cost of production, and make them available in clinics for diagnostic purposes. Sauer-Budge et al. used a high temperature resistant polymer and used more feasible fabrication techniques to allow the mass production of chips. The chip included a lysis and separation section to obtain nucleic acids, a zigzag mixing station to mix the reagents, an amplification region, and a fluorescence imaging section for detection [219].
2.4. Bacterial Behavior and Biomechanics

Mechanical properties of bacteria have been extensively investigated with scanning electron microscopy, atomic force microscopy, and with other techniques [220]. The sample preparation for these studies often requires fixation of microorganisms, which could distort the physical properties of bacteria. Microfluidic devices provide novel designs and techniques to entrap a single microorganism to study bacterial behavior, cell morphology, and biomechanical properties.

Mannik et al. observed the morphology of bacteria, in narrow channels, using a microfluidic system. This study, in particular, is critical for understanding bacterial cells in micro-scale confinements, as the bacteria were not forced into the narrow channels, but instead were attracted towards the channels via chemotaxis. They used silicon chips instead of PDMS, which may be advantageous due to its stiffness, but PDMS is more cost-effective since the same wafer mold can be used many times to obtain dozens of PDMS devices. The width of the features ranged from 5 μm to 0.3 μm while the depth was kept between 5-7 μm. Their results showed that E. coli with a diameter of approximately 0.8 μm could swim at its average speed (approximately 20 μm/s) when the width of the channel is larger than 1.1 μm. As the width became narrower, they stopped swimming, but still reached the chambers by dividing. Even at a width of 0.3 μm, cells were able to enter the chamber by deforming their structure. It is noted that they compared E. coli and B. subtilis and stated that B. subtilis were not able to enter channels narrower than their diameter as B. subtilis is a Gram-positive bacteria with a rigid cell wall structure [24]. Separately, Binz et al. investigated the motility of Serratia marcescens in narrow microfluidic
confinements and indicated that bacterial motility depends on microchannel size and geometry. Their results showed that complex geometries such as comb-structure stifle bacterial motility [221]. Furthermore, it has been demonstrated that channel geometry plays a very important role in bacterial movement. Galajda et al. changed the concentration of bacteria in microchambers using only funnel shaped entrances. They identified that the approach angle bacteria affects whether cells move along a surface or bounce back and swim away, resulting in a change in the concentration of bacteria at a region [222].

Sun et al. tested the stiffness of bacteria by using tapered microchannels. It was postulated that Gram-positive and Gram-negative bacteria have different stiffness properties due to their cell wall structures. This characteristic affects bacterial motility in narrow structures. Hence, they selected a Gram-negative (E. coli) and a Gram-positive (B. subtilis) bacteria with similar sizes. They flowed bacterial cells by applying pressure, and observed that E. coli could move further into tapered microchannels as compared to B. subtilis. Furthermore, the group exposed E. coli cells to an antibiotic that affects the shape of E. coli, and they stated that the antibiotic treated E. coli cells can move even further down the tapered channel when compared with the untreated ones [220]. These studies clearly highlight the potential of microfluidic devices to understand biomechanical properties of bacterial species.

In addition to these characteristics, it is observed that bacteria preferentially move at certain sides of the channels. DiLuzio et al. designed microfluidic channels that were made of agar on the bottom and oxidized PDMS on the sides and at the
top. By keeping the channel size narrow, they were able to track the movement of *E. coli* strains and concluded that there was a preferential movement of *E. coli* toward the sidewalls. The cells that swam toward the bottom of the channel and interacted with the agar moved toward the right, whereas the cells that occasionally swam at the top of the channel moved toward the left side [223]. Furthermore, Hill *et al.* stated that upon exposure to fluid shear, *E. coli* moved toward the left wall [224].

2.5. **Effect of Fluid Flow and Shear Stress on Bacteria**

The effect of fluid flow on bacteria is intriguing to researchers, as it is known that fluid flow and shear stress has profound impacts on bacterial growth, attachment, and biofilm formation. Many studies have been done to elucidate how fluid flow affects bacteria. Microfluidics offers a convenient way of testing the effect of flow and shear stress on bacteria. Rusconi *et al.* showed that biofilm formation is initiated by cellular streamers located at the corner of the microchannels under laminar flow [225]. Then, further investigation elucidated that depending on the angle of the microchannel, vortices could form at the corners, thus creating conditions favorable for biofilm formation [226]. Recently, they investigated the effect of shear stress on bacterial trapping, surface attachment, and chemotactic movement using both experiments and computational modeling. The authors concluded that bacterial species attach to surfaces, accumulate and promote biofilm formation at locations where there is high shear stress. This phenomenon is explained by the swimming behavior of bacteria, and their position within the channel. This could play a significant role in high bacterial infection rate in catheters. Furthermore, chemotactic movement decreased considerably at high-shear
stress regions [227]. The effect of fluid flow on microorganisms has also been shown by other studies [228, 229].

Kumar et al. designed a microfluidic device with features that resemble a shark skin to study the effect of secondary flow on bacterial biofilm formation. They showed that by incorporating a baffle geometry, fluid flow rate had an impact on where exactly the biofilm formation occurred. At high flow rates (8 µL/h) biofilm did not form in between the baffles whereas at slow flow rates (0.8 µL/h) biofilm formation was seen everywhere around the baffle. Additionally, as the baffle pitch was increased biofilm formation was observed inside the baffles even when the flow rate was high [230].

Understanding the bacterial adhesion process is critical since it is the first step of biofilm formation, and the starting point of microbial infections in humans [231]. Weaver et al. used human plasma fibrinogen to test the effects of shear stress on the adhesion of *Staphylococcus epidermis* to this protein. The PDMS microfluidic device contained four parallel circuits that allowed 4 different shear rates to be employed during the same experiment. Cell suspensions flowing through the device, at different shear rates, adhered to the surface and were analyzed with imaging software. The attachments were observed when the fibrinogen was intact. The results indicated that the adhesion of individual cells to the surface was favored at low shear rates (approximately 1 dyne/cm²) and decreased at higher shear rates. On the other hand, clusters of bacteria attached the best at a threshold shear rate (10 dynes/cm²), and below or above this value the adhesion was not favored. [232]. These results clearly indicate that bacterial adhesion depends on several things: flow regime, shear
stress, cell to cell, and cell to surface interactions. More understanding is critical in this area of research to unravel the role of flow regimes and bacterial interactions with surfaces.

2.6. Microfluidic Devices and Nanofabrication Techniques

Microfluidics is an excellent tool, and its advantages, characteristics, and applications were discussed in detail in the previous sections. Depending on the study, the fabrication techniques and the materials used to create these chips vary greatly. Materials from glass to elastomers are used to fabricate these devices, and each material has certain advantages and disadvantages. The application of these materials can be selected depending on the nature of experiments. In the following section, the properties and the fabrication techniques of glass/silicon chips and PDMS devices will be discussed in detail.

2.6.1. Microfabrication of Glass/Si and Etching Techniques

Glass and silicon are rigid materials that have been extensively used in industry. Due to their properties, they are preferred for processes that require high temperatures, high aspect ratios (width to height ratio), and low elasticity [233]. Fabrication of these chips takes a long time and is very costly whereas dozens of polymer microfluidic devices can obtained from a single fabricated master wafer. There are different fabrication techniques for glass/silicon, including bulk micromachining, and surface micromachining.

Bulk micromachining is extensively used in glass/silicon fabrication processes. The concept includes the removal or etching of the bulk substrate material to create the device. Both glass and silicon can be used for this process. It starts with
photolithography to pattern the desired design onto the wafer by using a UV-sensitive resist. This step creates a mask on the wafer surface to avoid the removal of specific areas during the etching process. There are mainly two types of etching procedures: wet etching and dry etching. Depending on the direction of etching, it can be called anisotropic or isotropic. Anisotropic etching does not occur at the same rate for each direction while isotropic etching etches the material every direction at the same rate (Figure 2) [234].

Wet etching uses chemical agents to selectively remove substrates. Many etchants including Ethyl diamine pyrocatechol (EDP), tetramethyl ammonium hydroxide (TMAH) and KOH lead to anisotropic etching, removing layers from vertical plane at a higher rate than from side planes whereas HNA (HF, Nitric acid, Acetic acid) is an isotropic etchant.
Dry etching is achieved by plasma etching or by reactive ion etching (RIE). Gas molecules are ionized at high voltage and used to remove the unmasked regions of the wafer. RIE and plasma etching are anisotropic. CF₄, SF₆, NF₃ and Cl₂ gases can be used to etch Si [234, 235].

A second technique for Si/glass fabrication is surface micromachining. As the name implies, these fabrication steps occur on the surface. A sacrificial layer that has the desired features is fabricated onto a wafer, and a structural layer is deposited.
on top of the sacrificial layer. The sacrificial layer is removed by etching to obtain the complete design [233]. The selection of the right structural and sacrificial material is important. Structural layers should be compatible with sacrificial layers. The materials must not react with the sacrificial layer and the removal procedure of the sacrificial layer must not demolish the structural layer [234].

2.6.2. Lithography Techniques

The word “Lithography” comes from Ancient Greek language, where *lithos* means “stones” and *graphia* means “writing” [236]. In microfabrication, lithography is used to create designs on substrates. Electron beam (e-beam) lithography, photolithography, ion-beam lithography and x-ray lithography are among the major lithography techniques. A lithography technique consists of three preliminary steps [237]:

- Initially, the substrate is coated with an irradiation sensitive resist, which is a polymer based chemical.
- Depending on the mask design, specific regions of the resist on the substrate are exposed to electron, ion and light beam.
- The resist is developed with a proper chemical to obtain the desired features.

Here in the following sections, e-beam lithography and photolithography will be discussed in more detail.

2.6.2.1. Electron Beam Lithography

Electron beam (e-beam) lithography uses electron beams to pattern the surface of the substrate with designed uploaded to a computer. This system is very
similar to Scanning Electron Microscopy with an addition of a blanker, which modulates the exposure of the surface to e-beam, and a pattern generator [238]. It is a widely used technique in nanofabrication since it provides very high resolution and very small features (down to 10 nm). One major drawback is that the technique does not offer high-throughput since the writing duration of the whole surface with e-beam is very long.

There are several factors affecting the quality and the speed of writing. Spot size, the e-beam energy and the dose, scattering of the electron (backward and forward), the choice and the thickness of resist, developer, and developing time [238]. Depending on the features and the resolution limit, poly-methyl methacrylate (PMMA), ZEP-520 and hydrogen silsesquioxane (HSQ) could be chosen as resists. Writing patterns could be achieved either by raster scan or by vector scan. Raster scan exposes the whole field of area regardless of the features whereas vector scan enables beam to move from pattern to pattern, skipping the remaining area on the field [239].

2.6.2.2. Photolithography

Photolithography, also known as optical lithography or UV lithography, is a lithography technique that uses UV light as the irradiation source to pattern features onto the substrates [240].

The fabrication process starts with drawing the channel design using a CAD program. Patterns can be drawn as separate layers on the program and used as individual masks in photolithography. For the features that require high-resolution, chrome masks can be chosen. Chrome mask is a type of photomasks often fabricated
with e-beam lithography [241]. Initially, chromium metal is deposited onto a glass/quartz substrate. Electron sensitive resist is spun onto the metal, and depending on the patterns, specific regions are exposed to e-beam. This mask contains necessary design to fabricate a master wafer with photolithography.

Depending on the complexity of the design, multiple layers can be achieved with photolithography. Both negative and positive UV-sensitive resists can be used for this process. Negative photoresist becomes cross-linked upon exposure to UV and unexposed parts are removed when the material is developed. Positive photoresist, on the other hand, is a cross-linked polymer and the exposure to UV breaks the cross-linked bonds, and these regions are removed upon developing the wafer. Factors affecting the feature quality include resist, baking time, exposure time, and developers.

Photolithography is much faster than e-beam lithography to create patterns, but the resolution is lower, where the smallest feature is 2 µm. Hence for features smaller than 2 µm, either higher resolution techniques need to be chosen, or photolithography should be followed by a wet etching step.

2.6.2.3. Soft Lithography

Soft lithography has become indispensable to microfluidics, particularly for biological studies. Devices made with this technique have been used for Polymerase Chain Reaction (PCR), cell capture, immuno-assays, DNA purification, and many more [242]. Many elastomers have been used in this technique such as Polyethylene glycol (PEG), PMMA, but PDMS is the most commonly used polymer in
microfluidics, and heat-cured on the master mold, which was created with other lithography techniques.

PDMS is an exceptional polymer for studying microorganisms. It is relatively chemically inert, an electrical insulator, and optically transparent for imaging [17, 242]. It is highly permeable to air but only moderately permeable to water, thus providing an excellent oxic and moist environment for bacterial growth [16, 17, 242-244]. The preparation procedure for PDMS is very straightforward and multiple devices can be molded from the same master wafer. This soft lithography process is very economical compared to wafer microfabrication.

Another important characteristic of PDMS is that it can be bonded to surfaces. It either forms reversible bonds via Van der Waals interactions or permanent covalent bonds between silicon and oxygen upon plasma treatment [242]. This property comes from the chemical structure of PDMS. The chemical formula reveals a very hydrophobic surface with a high number of methyl groups connected to the siloxane backbone as can be seen from Figure 3 [234]. The hydrophobic surface can be converted to a hydrophilic one with oxygen plasma etching, replacing the methyl groups (-CH₃) with hydroxyl groups (-OH) [245]. PDMS maintains its hydrophilic properties as long as it interacts with water; otherwise it changes back to its hydrophobic state [242].
Figure 3: Chemical formula of Poly-dimethylsiloxane (PDMS).

The elastic properties of PDMS present both benefits and drawbacks. Young’s Modulus determines the elasticity of PDMS, which can be modulated by the mixing ratio of base and the curing agent, curing time, and temperature [246]. Elasticity gives versatility to PDMS as valves and mixers can be made and added as additional layers [242]. The Young’s modulus allows large deformability of the elastomer. PDMS has a moderate modulus that is around 2.8 MPa [247]. This number becomes very important in channel collapse problems. Channel collapse occurs when the roof of the PDMS microfluidic channel adheres substrate, which can be glass or another PDMS substrate [248]. The aspect ratio (width:height) of the channels is an important factor in channel collapses. Huang et al. and Zhou et al. studied the factors that cause roof collapse in PDMS, and obtained a series of equations for collapse criteria [247, 249]. They developed an equation to evaluate the “no collapse region”:

$$\frac{4ay}{E'h^3} < 0.83 \ (1)$$

where: 2a = Punch spacing (µm)

$$\gamma = \text{Work of adhesion} \ (\text{mJ/m}^2)$$
$E' = \text{Plain-strain modulus (MPa)}$

$$E' = \frac{4}{3} E \quad (2)$$

where: $E = \text{Young’s modulus (MPa)}$

$h = \text{Punch height (\text{\textmu}m)}$
3.0 Research Aims

Four aims are investigated in this thesis. In the following sections, each aim will be introduced, and their methodology and results will be discussed in detail.

**Aim 1:** Design and fabricate a novel and passive polymer sub-microfluidic device with size-specific constrictions to separate bacterial cells from heterogeneous bacterial cultures:
- Identify the design parameters for microfluidic devices to successfully separate bacterial cells.
- Perform and optimize microfabrication processes in cleanrooms to achieve sub-micrometer to nanometer size constrictions.
- As a proof-of-concept, self-sort two model microorganisms, *Pseudomonas aeruginosa* and *Escherichia coli* mixed at varying concentrations.
- Demonstrate that the device could randomly separate species with similar sizes by using GFP *Escherichia coli* and m-cherry *Escherichia coli*.
- Perform the initial tests for culturing and separating a mixture of two previously uncultivated environmental species: *Psychroserpens* sp. and *Roseobacter* sp.
- Analyze the diffusion of Lysogeny Broth from an isolation chamber to the main entrance through a constriction by computational modeling.

**Aim 2:** Design and fabricate a prototype sub-microfluidic device for *in situ* isolation and cultivation of species from environmental and patients’ samples.
- Create a protocol to implement nanoporous membranes onto the sub-microfluidic devices to facilitate transport of nutrients and growth factors from the environment into the isolation chambers to induce the growth of isolated species.
- Apply these devices for *in situ* isolation, cultivation and identification of species from different environments, including the Charles River in Boston, MA, a lake in Greenland, and human fecal samples.
- Perform computational and empirical analyses of the diffusion rate of small molecules from the environment into the isolation chambers of the sub-microfluidic devices through nanoporous membranes.

**Aim 3:** Investigate bacterial behavior in confinements with varying sizes under applied pressure in order to understand the effectiveness of water filtration processes.
- Perform control experiments with fluorescent polystyrene beads.
- Create pressure profiles for several microorganisms in confinements with varying sizes.
- Analyze the flow mechanics inside the microfluidic device using computational modeling.

**Aim 4:** Create a sub-microfluidic platform to examine the bacterial behavior in the presence of different nutrients and antimicrobial agents.
- Perform antimicrobial susceptibility tests on microorganisms using sub-microfluidic devices with constrictions.
- Develop a protocol to couple a sub-microfluidic device to Surface Plasmon Resonance imaging (SPRi) to observe bacterial response to chemicals in real time.
4.0 Methodology

4.1. Aim 1

4.1.1. Design and Fabrication of the Master Wafer

Constrictions and large microchannels were designed as separate mask layers using Tanner L-Edit version 15.1 software (Figure 4). Several distinctive designs with varying widths, lengths, heights and geometries were investigated to determine the optimum conditions for capturing bacteria. The first design includes 950 nm tall constrictions with widths ranging from 1.5 μm to 5 μm. The lengths were varied from 20 μm to 40 μm. The second design had constrictions that were 700 nm tall, 0.75-2 μm wide and 20-40 μm long. Isolation chambers were 100-200 μm wide, 7.5 μm tall, and 2-4 mm long while the main entrance is 100 μm wide, 7.5 μm tall, and 19 mm long. Additionally, triangle, circle, square and tapered constrictions were also designed to test the bacterial capture efficiency. The dimensions of the main entrance and the isolation chambers were significantly larger compared to the size of bacterial cells and provide adequate space for normal cellular functions such as swimming and growth. Scanning Electron Microscope (SEM) images in Figure 5 (A,B) provide a closer look at the master wafer containing isolation chambers connected to the constrictions and illustrate distinct constriction designs. Also, Figure 6 demonstrates the brightfield images of the two different constriction designs fabricated on a master wafer.
Figure 4: A master wafer design drawn with Tanner L-Edit. It includes 8 different microfluidic chips with large isolation chambers and a main entrance on a 3-inch Si wafer. (Constrictions are not visible at this magnification).
Figure 5: (A, B) SEM images of a completed wafer. (A) One large isolation chamber is connected to a sub-micron constriction. (B) The main entrance is connected to 3 isolation chambers via square and circular constrictions.

Figure 6: (A, B) Brightfield images of the master wafer containing different constriction designs. (A) A straight constriction. (B) A tapered constriction design.

In order to fabricate the master wafer, the first approach was to pattern the constrictions with a photolithography step and adjust their width by using chromium etchant. Prior to this, a chromium metal was sputtered onto a clean 3-inch Silicon wafer at a desired thickness. As mentioned in the Critical Literature Review Section, the etching step is anisotropic as shown in Figure 2.
The second, and a more effect, approach was to pattern the constrictions using electron beam lithography process. First poly-methyl methacrylate resist (PMMA 950 A9, MicroChem) was spun on a clean p-doped 3-inch Silicon wafer (University Wafers) at 3000 rpm for 1 minute (Laurell Spinner) and baked at 175 °C for 5 minutes. The thickness of the resist was confirmed with an optical profilometer (Nano-Spec) as 1.3 μm. The constriction design was defined on the PMMA using a dedicated electron-beam writer (Elionix F-125) at 125 kV with a current of 10 nA and dose of 1800 μC/cm². After exposure, the wafer was developed in 3:1 (v/v) ratio of isopropanol to methyl isobutyl ketone (MIBK) for 90 seconds and rinsed thoroughly with isopropanol (IPA). Chromium metal sputtering was achieved with a magnetron metal sputterer at 500 W and 275 DC Volts (MRC 8667 Sputtering). Lift-off was done with acetone. The thickness of the metal on the wafer was confirmed with a surface profiler instruments (DEKTAK Profilometer 3ST, Zygo NuView 6000 Optical Profilometer). Next, positive photoresist (Clariant AZ4620) was spun at 3000 rpm for 1 minute (Laurell Spinner) and baked for 1 minute at 115 °C. A 4” x 4” chrome mask for larger microchannel features was ordered from the Front Range PhotoMask Co., and the mask was aligned to the constrictions using photolithography. The photoresist was exposed to the UV light for 45 seconds (Quintel 4000 Mask Aligner). The features were developed in a 3:1 (v/v) ratio of deionized water to AZ400K developer. At the end of this step, a completed master wafer was obtained. A schematic of the device fabrication protocol is demonstrated in Figure 7.
Figure 7: Schematic of a PDMS microfluidic device fabrication. 1) PMMA is spun on a 3 inch Si-wafer. 2) The constriction pattern is created with electron beam lithography and developed. 3) Chromium is sputtered (height of the constriction is determined by this step). 4) Lift-off is done. 5) Positive photoresist is spun. 6) Large features are aligned over the constrictions and created with photolithography. 7) Large features are developed, completing the master wafer. 8) PDMS is molded. 8) PDMS is peeled off, access holes are drilled. 9) The device is bonded to a glass cover slip using oxygen plasma (adapted from ref [250]).
4.1.2. PDMS Devices

To create patterned PDMS polymer microfluidic devices from a master wafer, the base and the curing agent were mixed together in a 10:1 (w/w) ratio (Sylgard 184 Silicone Elastomer Kit, Dow Corning). The master wafer was vapor coated with chlorotrimethylsilane (CTMS) (Acros Organics) to allow easy separation of the polymer from the master after heat-curing. The PDMS mixture was poured onto the wafer, degassed in a vacuum chamber until all the air bubbles were removed, and heat-cured at 70 °C for 3 hours. The cured PDMS, which contains the negatives of the features found on the master wafer, was peeled from the master and cut into sections, where each section contains one device. SEM images of the master wafer and PDMS devices were taken with Hitachi S-4800 Field Emission Scanning Electron Microscope. Prior to SEM imaging, the PDMS pieces were sputter coated with a thin layer of Pt/Pd alloy using a Cressington Sputter Coater 208HR to make the samples conductive.

Access holes were drilled into the isolation chambers and the main entrance manually using a sharpened 18G needle (Small Parts). The patterned side of the PDMS was permanently bonded to a microscope cover slip (Fisher Scientific, microscope cover glass, 0.17-0.25 mm thick) using oxygen plasma (Anatech SP-100 Plasma System) at 100 W, 0.4 Torr for 5 seconds. The completed microfluidic device is shown in Figure 8.
Figure 8: Photograph of a completed PDMS sub-microfluidic device.

4.1.3. Bacterial Cultures

M-cherry red fluorescent protein (RFP) labeled *E. coli* DH5-α strain was cloned with a pUC19 plasmid at high copy number while green fluorescent protein (GFP) labeled *E. coli* DH5-α strain contains a high copy number of pLIT 29 plasmid (both strains were kindly supplied by the Godoy Lab, Northeastern University-Department of Biology). The lac promoter in both strains regulates the expression of the fluorescent protein gene. An ampicillin resistance gene was inserted into the plasmid to ensure the selection of fluorescent protein labeled bacteria from LB-agar plates (BD, Difco LB, Miller and BD, Difco agar, Technical) containing ampicillin (Sigma Aldrich, ready-made ampicillin). Cyan fluorescent protein (CFP) labeled wild-type *P. aeruginosa* PAO1 strain was kindly supplied by the Epstein Lab, Northeastern University-Department of Biology. The CFP gene was directly inserted
into the genome of the bacteria so is expressed constitutively. Both *P. aeruginosa* and the marine strains were grown in LB-agar plates.

Prior to loading the device with bacterial mixture, both *E. coli* strains were cultured overnight at 37°C in 6 mL of LB-ampicillin at a 1000:1 (v/v) ratio. *P. aeruginosa* and the marine species were cultured overnight in 6 mL of LB at 37°C and room temperature, respectively. All liquid cultures, except the marine species were incubated on a shaker adjusted to 127 rpm [250].

**4.1.4. Experimental Procedure and Fluorescence Imaging**

Food chambers were filled with LB from the access holes using a 19 Gauge needle connected with tubing to a filled syringe as can be seen in Figure 9. The main entrance channel was filled with Phosphate Buffered Saline (PBS) using the same approach. Filling of the device was monitored by eye.

Two bacterial strains were separately cultured overnight in LB and the cellular concentration of each culture was determined using a hemocytometer and adjusted to obtain the target cell ratio at the start of each experiment. Depending on the experiment, 1:1 (v/v), 2:1(v/v) and 1000:1(v/v) aliquots of the two adjusted bacterial cultures were collected and thoroughly mixed. A droplet of culture was inoculated at the main entrance of the main entrance. The device was incubated overnight at 37 °C in humid conditions. The device containing marine species was incubated at room temperature.
Figure 9: (A-D): Experimental procedure for filling the PDMS microfluidic device. (A) All the isolation chambers were filled with LB while the main entrance was filled with PBS. (B-C) Bacteria were separately cultured on agar plates, and grown in 6 mL of growth media. (D) A bacterial culture was prepared by mixing two species and a droplet of this mixture was inoculated into the access hole of the main entrance.

All images were taken with a Zeiss Axio Imager A2 fluorescence microscope at 40X lens, and 100X oil immersion lens with a 1.3 numerical aperture objective. The microscope contained both GFP and RFP filters for fluorescence imaging. Fluorescence light was provided by X-Cite Series 120Q excitation light source. Zeiss
Axiovision version 4.8.2.0 imaging software was used to process the images obtained with an AxioCam MRm CCD camera [250].

4.1.5. Computational Modeling of the Device

The diffusion rate of nutrients is critical to attract bacteria towards the constrictions. The device performance was analyzed by using the transport of dilute species model in COMSOL 4.2 Multiphysics software. The modeled design contained a single 7 µm tall, 200 µm wide, 4 mm long isolation chamber that was connected to a large reservoir of water via a 1 µm tall, 1.5 µm wide, 40 µm long constriction. Two different models were constructed for analyses. The first model contained 8.8 mM of leucine in the isolation chamber and the constriction while the second model utilized 5.1 mM of serine. The concentrations of both amino acids in LB were based off of literature values [251].

4.2. Aim 2

4.2.1. Microfabrication of the Device

The microfabrication process of the devices for Aim 2 follows a very similar protocol to Aim 1, which was explained in detail in Section 4.1.1. A few adjustments were made to the described procedure. Sharpened 14G needles were used to drill access holes to the isolation chambers and the main entrance. The height of the isolation chambers and the main entrance were modified to 2-3 µm, instead of 7.5 µm, which was confirmed with a stylus profiler. To achieve this, a different positive photoresist, AZ 1827, was used to create a thinner resist layer. Additionally, the PDMS devices were obtained by preparing 5:1 (w/w) ratio of the PDMS base to the
curing agent instead of the conventional 10:1 (w/w) ratio used in microfluidics research in order to decrease the elasticity of PDMS.

4.2.2. Incorporation of Nanoporous Membranes onto the PDMS Device

Two nanoporous membranes were selected to cover the access holes of the isolation chambers: nucleopore track-etched polycarbonate (PC) membrane (0.05 µm pore size, 25 mm diameter, Whatman) and aluminum oxide (Anodisc®) membrane (0.02 µm pore size, 13 mm diameter, unsupported, Whatman). The membranes were sputtered with a 3 nm layer of Pt/Pd alloy, and their SEM images were taken to confirm their porosity and the pore sizes.

For the in situ experiments, all the access holes of the isolation chambers were covered with a PC or a Anodisc membrane and sealed around the edges of the membrane-PDMS interface by using a waterproof silicone glue (Master Tradesman Indoor/Outdoor Window/Door Caulk) while keeping the access hole of the main entrance open (Figure 10). For the negative controls, all the access holes of the isolation chambers and the main entrance were covered and sealed with a PC membrane. The devices were then placed into the bottles containing a mixed culture of P. aeruginosa, E. coli, and Staphylococcus aureus (S. aureus). The devices were incubated for two days at room temperature before taking brightfield images.
4.2.3. *In situ* Experiments

4.2.3.1. Human Fecal Samples

Fresh human fecal matter was obtained from a healthy donor within 20 minutes after passage by using a Medline 2-piece specimen collection kit (Medline, DYND36500). For the conventional plate technique, the human fecal sample was prepared by diluting 1 g of stool in reduced PBS with 0.1% cysteine for $10^6$ times.
The fecal slurry for the devices was prepared by diluting 1 g stool in Gifu anaerobic media (SGAM) for $10^6$ times [252, 253].

150 mm diameter petri plates (Fisher Scientific) were inoculated with 250 µL of fecal slurry and spread plated using a sterile cell spreader for the conventional plating. The plates were incubated for 7 days at 37 °C in the vinyl anaerobic chamber (Coy Labs, Type B vinyl anaerobic chamber). Individual colonies were randomly picked from the plates and streaked on petri plates prepared with SGAM until a single isolate was obtained. Liquid media stocks were created with 25% glycerol and 0.1% L-cysteine for sequencing 16S rRNA genes through Sanger sequencing.

The PDMS devices and the membranes were both autoclaved prior to running the experiments. The devices were filled with SGAM and then the PC membranes were sealed over the access holes of the isolation chambers with the waterproof silicone glue. The devices were placed into 100 mL sterile glass bottles that contains 45 mL of fecal slurry and were incubated for 7 days at 37 °C in the vinyl anaerobic chamber. After the incubation, the isolates were collected from each isolation chamber and spread plated onto SGAM plates. Colonies that demonstrated distinct morphologies on plates were cultured again until a single isolate was obtained. Liquid media stocks were prepared for 16S rRNA gene sequencing.

Similar to the 16S rRNA sequencing of the river samples, the genomic DNA obtained from the isolates was initially amplified with PCR. 27F and 1492R universal primers, Phusion® High-Fidelity DNA Polymerase (NEB M0530) and 5 x
Phusion® HF® Buffer (NEB B 0518) were used for PCR. The recipe for 30 cycles of PCR amplification was as follows:

1. 98 °C 30 s
2. 98 °C 10 s
3. 52 °C 30 s
4. 72 °C 60 s
5. Go to 2 30X
6. 72 °C 5 min
7. 10 °C hold

Post PCR clean up was performed with AMPure SPRI beads by following the manufacturer’s protocol (Beckman Coulter). For the Sanger Sequencing, extraction was done with Peg-200 lysis buffer (Sigma Aldrich) made alkaline (pH 13.5) with 1.86 mL of 1M KOH and 39 mL of water. 2 µL of liquid stock (25% glycerol and 0.1% L-cysteine) for each isolate with 20 µL of lysis buffer was heated at 90 °C for 10 minutes. 27F Universal Primer was used for Sanger sequencing.

4.2.3.2. River Samples

The river water was collected from the Charles River in Boston, MA once in February 2015, and once in July 2015 to isolate, and identify species grown in the river, and to observe the distribution of species at different seasons. To avoid spatial variations, the water sample was collected from the same location each time. The devices were first filled with sterile deionized water and sealed with a polycarbonate (PC) or an Anodisc membrane over the access holes of the isolation chambers with silicone glue. Once the glue was dry, the devices were placed into the sterile bottles.
that were filled with the river water (Figure 11). The winter samples were incubated at 4 °C whereas the summer samples were incubated at room temperature for one month.

Figure 11: The PDMS sub-microfluidic device with an Anodisc membrane covering all the access holes of the isolation chambers while leaving the main entrance was incubated in a bottle filled with the river sample. The accumulation of bacteria on the membrane could be seen from the yellowish color change on top of the membrane.

After incubation, the devices were collected and observed under an optical microscope. Samples were collected from each isolation chamber and spread plated onto the 5X diluted LB-agar plates. The grown colonies were picked and cultured in 5X diluted LB and were sent for 16S rRNA gene sequencing.

16S rRNA sample preparation and sequencing was performed on bacterial colonies by Macrogen Corp in Maryland, U.S.A. Initially, genomic DNA (gDNA) was isolated from liquid cultures, followed by PCR amplification with universal primers and by PCR purification. The primers are:
27F (-AGAGTTTGATCMTGGCTCAG)
1492R (-TACGGYTACCTTGTTACGACTT)

Amplified gDNA was sequenced with Sanger sequencing method (BigDye® v3.1, Life Technologies, Applied Biosystems) by following the manufacturer’s guidelines. Sequencing was performed with both Forward (518F) and Reverse (800R) universal primers. Sequence detection was conducted by capillary electrophoresis on a 3730xl Genetic Analyzer (Life Technologies, Applied Biosystems) using a 50 cm array, the Long DNA sequencing module (LongSeq50_POP7) and the KB analysis protocol (KB basecaller) with the default instrument settings. Post-detection, raw signal data was initially processed on the 3730xl Genetic Analyzer computer using Sequencing Analysis v5.3.1 (Life Technologies, Applied Biosystems).

The chromatogram of each forward and reverse sequence was individually analyzed, and bases with Phred quality scores lower than 30 were trimmed automatically by using a sequence analysis program (CLC MainWorkbench 7.6.3, Qiagen). Consensus sequences were obtained by assembling forward and reverse trimmed sequences with medium alignment stringency. Minimum alignment read length was set to 50, and upon conflicts between the nucleotide reads of the two sequences, the most seen nucleotide was used.

Consensus sequences were uploaded as query sequences into the BLAST and RDP (Ribosomal Database Project) databases, and the sequences were matched and classified in both database. When determining the classification of the
microorganism, the sequence similarity threshold was set to 97% and the results from each database together with their chromatogram data were compared.

4.2.4. Diffusion Studies

The mass transfer of molecules through the PC and Anodisc membranes was studied both empirically and by computational modeling. The diffusion coefficient of 10 µM pyocyanin (Sigma-Aldrich) in 10 mM PBS was determined empirically using a disposable, screen-printed electrochemical sensor (TE100, Zensor). The sensors utilize a 3-electrode setup containing carbon-based working (3 mm diameter disk) and counter electrodes along with a Ag/AgCl reference electrode [254]. All electrochemical measurements were recorded with a potentiostat (CHI1040C, CH Instruments). A 150 µL of pyocyanin solution was placed over the sensor surface and the oxidation/reduction of pyocyanin was measured using cyclic voltammetry at different scan rates.

The electrochemical detection of pyocyanin concentration was initially performed without a membrane as a control and with either a PC or an Anodisc membrane placed on top of the access holes of the isolation chamber. To mimic this setup, a 2.5 mm diameter, 3.5 mm tall PDMS well was bonded to an electrode sensor using oxygen plasma. The setup can be seen in Figure 12 (A-C). The measurement was performed for 50 minutes.
Figure 12: (A–C) The setup for the electrochemical detection of pyocyanin using an electrochemical sensor. The PDMS well was filled with PBS solution and a droplet of pyocyanin was placed on top of the well when: (A) A PDMS well with no membrane (B) A PDMS well covered with a PC membrane. (C) A PDMS well covered with an Anodisc membrane.

Computational modeling studies were conducted using the diffusion of dilute species module in COMSOL Multiphysics 4.4 Software. In order to simulate pyocyanin diffusion through the membrane-PDMS interface, a 3D model having the same dimensions as the empirical PDMS device was designed. Diffusion simulations modeled the diffusion rate of 10 μM of pyocyanin into the isolation chamber without a membrane and either through a PC or Anodisc membrane. There is no convection in the system, therefore the mass transfer of pyocyanin occurs only via diffusion. Although the system is not stationary, diffusion through the membrane can be assumed to be pseudo-steady state since the membrane is very thin. The porosity of the membranes was modeled by calculating the total porous area covering the access
hole of the isolation chambers and representing it as 10 or more pores in the model. The isolation chamber was modeled as a tall cylinder and the pyocyanin droplet was also modeled as a cylinder on top of this system to create the wetting effect occurring as the droplet contacts and disperses along the hydrophilic membrane.

4.3. Aim 3

Microfluidic devices with 950 nm and 700 nm tall constrictions were used to investigate the bacterial behavior in pores under applied pressure. Initially, fluorescent polystyrene beads with a diameter of 1 µm were used as a control. For the bacterial studies, CFP *P. aeruginosa* PAO1 strain, m-cherry *E. coli*, *S. aureus*, and the marine species *Psychroserpens* sp. were used. All cultures were prepared fresh in 6 mL of LB and incubated overnight, except that m-cherry *E. coli* was cultured in 6 mL of LB-amp. *Psychroserpens* sp. was cultured at room temperature while the other species were cultured at 37 ºC.

Prior to the experiment, both beads and the bacterial culture were diluted 2X with LB (~10 cells/µL final concentration) in order to avoid clogging of the pressure inlet channel and the entrance of the constriction. The pressure head was adjusted with a microfluidic flow control system (Fluitgent, MFCS 4C) connected to a house airline. The system was directly connected to the inlet of the sub-microfluidic device (Figure 13). After applying each pressure value, images of the constriction were taken with an optical microscope. The pressure at which the first cell entered the constriction was recorded, and the pressure profiles were created for each microorganism.
Figure 13: (A) The PDMS microfluidic device. (B) The schematic of one part of the microfluidic device with a sub-micrometer constriction attached to the constriction. A precise pressure head was applied at the inlet using a microfluidic flow control system. The outlet channel is open to the atmosphere (adapted from ref [255]).

4.4. **Aim 4**

Initial studies were conducted on the effect of penicillin/streptomycin on *S. aureus*. The bacteria were cultured in 6 mL LB overnight at 37 °C. Penicillin/streptomycin cocktail was prepared with 10000 units/mL penicillin and 10000
µg/mL of streptomycin and the cocktail was diluted 1000X and 200X in LB. The antibiotic cocktail was loaded into the isolation chambers and the constrictions, and the main entrance were flushed with LB a twice to remove the entire antibiotic from the main entrance and filled with LB. A droplet of S. aureus culture was inoculated into the access hole of the main entrance and incubated overnight at 37 °C.

In order to permanently bond sub-microfluidic devices on the gold prism of the SPRi, (3-mercaptopropyl)trimethoxysilane (MPTMS) (95%, Sigma Aldrich) was used as a thiol-silane linker. For initial bonding tests, a microscope slide was sputtered with gold by using electron beam evaporator. 25 mM and 5 mM MPTMS solutions were prepared by adding 46 µL and 9.6 µL of 95% of MPTMS in 10 mL of 200 proof Ethanol (Sigma Aldrich), respectively [256]. Gold-coated glass slides were initially cleaned with acetone and isopropyl alcohol, and then were dipped into the MPTMS solution for 1-3 hours. After the treatment, the glass slides were rinsed with absolute ethanol. The PDMS devices were plasma treated for 5 seconds at 100 W, 0.4 Torr, and bonded to the MPTMS treated glass slides. The PDMS device-gold coated glass slides were post-baked at 70 °C for 1 hour to enhance the bonding, and placed onto the gold prisms by using a refractive index matching oil.
5.0 Results and Discussion

5.1. Aim 1

5.1.1. Microfabrication of the Master Wafer and PDMS Devices

The key factor of this aim is to accurately design and fabricate the constrictions with precise geometries. Before using e-beam lithography as a fabrication step, the constrictions were fabricated with a 2-step photolithography procedure that involved sputtering of a 700 nm thick Cr metal on a 3-inch p-doped Si-wafer first. One major constraint of UV photolithography is the resolution limit, which is excellent for features down to 2 µm. A wet-etching process was therefore required at the end of the first photolithography step to achieve constrictions narrower than 2 µm. Chromium etchant was used as the wet-etchant in this study, which etches the chromium metal anisotropically as mentioned in Critical Literature Review. Etching rate varies greatly depending on the temperature, the shaking rate during etching, and the oxide thickness on the wafer. Due to these variations, etching caused rough uneven structures both on the wafer and on the PDMS, which can be seen in Figure 14.
Figure 14: (A, B) SEM images of a constriction after treated with Chromium etchant (A) The constriction on the wafer (B) The constriction on the PDMS.

While the wet-etching technique led to constrictions narrower than 2 µm, creating well-defined and smooth constrictions is very critical for size-specific separation of bacterial cells. This method was therefore not suitable for creating these structures.

E-beam lithography, which is an alternative method to fabricate submicrometer constrictions, provides excellent resolution properties, reaching as low as 10 nm [257]. The process does not require any wet-etching step. It can be clearly seen from the SEM images of an e-beam patterned wafer and the PDMS obtained from that wafer in Figure 15 that the constrictions are very smooth and sharply defined. Hence, the first optical lithography step to build constrictions was eliminated, and replaced with the e-beam lithography step. The major issues with this technique are the optimization of the applied electron-beam dosage and subsequent lift-off procedure. We initially started e-beam writing with a dosage of 1500 µC/cm² and increased it to 1800 µC/cm² after considering the size of the
features and the total area to be written. The relation between dose, area, time, and current can be shown in an equation:

\[ D \times A = t \times I \] (3)

where:

- \( D \) = Dosage (\( \mu \text{C/cm}^2 \))
- \( A \) = Total area (\( \text{cm}^2 \))
- \( t \) = Time (seconds)
- \( I \) = Current (A)

In order to hinder the movement of bacteria inside the constrictions, some of the constrictions were designed smaller than the size of bacteria at least in one dimension, and some smaller both at height and width. This approach in product design significantly improved the success of the bacterial separation process.
Figure 15: SEM images of one isolation chamber that is connected to the main entrance with a sub-micrometer constriction. (A, B) SEM images of the fabricated master wafer. Constrictions were fabricated with electron-beam lithography to obtain exact widths at high precision in a single step. (C, D) SEM images of the PDMS device. (D) The magnified image of the constriction shown in (C). White particles seen on the SEM images formed during the metal sputtering step of SEM sample preparation. Devices used in experiments do not have any visible particles on the surface (adapted from ref [250]).

During the soft lithography step, one main challenge that many groups, including us, who work with sub-micron channels, experience is the channel collapse. When the PDMS chips were irreversibly bonded to glass cover slips, constrictions often collapsed as shown in Figure 16. To solve this problem, the ratio of the PDMS mixture and the baking time was changed to reduce the elasticity of PDMS. Increasing the baking time to 3-5 hours from 1 hour at 70 °C significantly improved the collapsing issue. The variations in plasma etching parameters also
affect the adhesion energy of PDMS and the glass cover slip, and the collapsing problem.

It is important to note that during the design process of microchannels, the yield of uncollapsed constrictions significantly increased when the aspect ratio of the constrictions to the isolation chamber and the main entrance was kept at 1:3 and 1:4, instead of 1:10. One hypothesis regarding this observation is that the larger channels might be acting as a beam, and supporting the sub-micron structures better. Further analysis is necessary to investigate this behavior.

![Figure 16](image)

**Figure 16:** (A-C) Optical images of permanently bonded PDMS. (A) Uncollapsed constriction. (B) Partially collapsed constriction. (C) Fully collapsed constriction.

### 5.1.2. Separation of Bacterial Cells

#### 5.1.2.1. CFP *Pseudomonas aeruginosa* and M-cherry *Escherichia coli* Cultures

The initial studies were carried out with model microorganisms: *P. aeruginosa* and *E. coli*. *P. aeruginosa* is a very motile bacteria that can go as fast as 40-50 \( \mu \text{m/s} \). It is rod-shaped, and Gram-negative bacteria with an average diameter of 0.8 \( \mu \text{m} \). *P. aeruginosa* forms robust biofilms, and is a pathogen for immunocompromised patients [28, 258, 259]. *E. coli* is also a rod-shaped, Gram-negative bacteria with an average diameter of 1 \( \mu \text{m} \). *E. coli* swims with a speed of
Both strains were modified to express fluorescent protein to facilitate their identification although the devices do not require labeling for detecting bacteria.

Both species were initially mixed at a 1:1 (v/v) ratio after reaching stationary phase of growth and after their concentrations were adjusted. Hemocytometer counts after 22 hours of cultivation at 37 °C indicated that CFP *P. aeruginosa* culture contained approximately $6.04 \times 10^7$ cells/mL while m-cherry *E. coli* and GFP *E. coli* cultures had $5.97 \times 10^8$ cells/mL and $2.75 \times 10^7$ cells/mL, respectively.

The chemotactic movement of the bacteria toward the constrictions was facilitated by filling the isolation chambers with LB, and the main entrance with PBS. Results elucidated that the bacterial cells easily passed through the constrictions wider than their own size (2 μm and wider) (Figure 17). It is important to note that though the constrictions were wide, bacteria were still separated from each other by forming multiple files inside the constriction. Intra-species interactions might be playing a substantial role in this behavior, which was also reported by other studies [159, 262]. Further investigation might be necessary to understand this phenomenon.
Figure 17: Isolation of CFP *P. aeruginosa* via a 950 nm tall, 2.5 μm wide, and 20 μm long constriction. (A) GFP filtered image of the device. CFP *P. aeruginosa* formed 2 lines inside the constriction. (B) RFP filtered image of the device. M-cherry *E. coli* could not enter the constriction or the isolation chamber (adapted from ref [250]).

1.5 μm wide constrictions trapped the bacteria into the constrictions, and regardless of the height of the constrictions, no bacteria were observed when constrictions were narrower than 1.5 μm. When the width was kept constant at 1.5 μm and the height was varied between 700 nm and 950 nm, fluorescence images in Figure 18 showed that both constrictions trapped *P. aeruginosa*. However, 700 nm tall constrictions more effectively impeded bacterial movement, and caused deformation in bacterial shape without affecting their viability. The length of constrictions did not have any effect in sorting.
Figure 18: (A-D) Fluorescence images of CFP *P. aeruginosa* PAO1 and m-cherry *E. coli* in a device with 1.5 µm wide and 700 nm tall constriction. (A) 40X GFP filtered image. *P. aeruginosa* formed a single line in the constriction and reached the isolation chamber. (B) 100X GFP filtered image of the same constriction. (C, D) 40X and 100X RFP filtered images of the same device, respectively. The isolation chamber and the constriction in both images are dark due to the absence of *E. coli* while the presence of the bacteria can be seen in the main entrance (adapted from ref [250]).

Images showed that when a heterogeneous culture of *P. aeruginosa* and *E. coli* was introduced into the microfluidic device, often *P. aeruginosa* reached the constrictions much faster than *E. coli* did, formed a biofilm, and blocked *E. coli* from entering the isolation chamber. In order to investigate how the concentration of bacteria in heterogeneous culture affects the sorting, the ratio of microorganisms in the culture was varied from 1:1 to 2:1 and 1000:1 (v/v) *E. coli : P. aeruginosa*. Results demonstrated that even though more *E. coli* cells were present initially, the
*P. aeruginosa* population always outgrew (Figure 19 and Figure 20). This could be because *P. aeruginosa* produces toxins such as pyocyanin that act as antimicrobial agents against other bacterial species [250].

![Figure 19: (A-B) GFP and RFP filtered images of 2:1 (v/v and concentration) ratio of m-cherry *E. coli* : CFP *P. aeruginosa* in a device with 700 nm tall, 1.5 µm wide constriction, respectively. (A) CFP *P. aeruginosa* entered the isolation chamber by forming a visible single file in the constriction (B) m-cherry *E. coli* were present in the main entrance, but their concentration was much lower than *P. aeruginosa* even though their initial inoculum concentration was higher than *P. aeruginosa* (adapted from ref [250]).]
Figure 20: (A-B) GFP and RFP filtered images of 1000:1 (v/v and concentration) m-cherry E. coli : CFP P. aeruginosa in a device with 950 nm tall, 1.75 µm wide constrictions, respectively. (A) CFP P. aeruginosa entered and populated the isolation chamber preventing E. coli from entering. P. aeruginosa in the main entrance and in the constriction were not visible using fluorescence microscopy due to photobleaching. (B) m-cherry E. coli were present in the main entrance, but could not enter the isolation chamber (adapted from ref [250]).

The experiments were repeated multiple times. The success rate of isolating individual species in isolation chambers diminished with increasing constriction widths. 49 constrictions with widths varying from 1.5 µm to 5 µm, and with a height of 950 nm were tested using a mixture of P. aeruginosa and E. coli (Table 1). A single species was observed in 22 of the 49 isolation chambers. 11 of 21 constrictions with widths ranging from 1.5 µm to 2 µm had cells formed a single file, as shown in Figure 18, and only contained one species in the isolation chambers. 10 of the 21 constrictions in this width range had no growth in the constriction. The data for the 21 constrictions are ideal as all of the isolation chambers contained 1 or 0 species.

28 constrictions were tested that had a width 2.5 µm or greater. 11 of which had multiple lines of cells, but still contained a single species in the isolation
chamber, similar to the results shown in Figure 17. 7 isolation chambers connected to these constrictions contained both species while 10 remained empty. The separation efficiency decreases for constrictions having width 2.5 µm or greater, but is still quite good at 39%.

20 chambers showed no growth inside, and 16 of them were blocked by dust or air bubbles, thus prevented cells from entering. Compared against other currently used colony isolation techniques, this separation efficiency is very promising. 20 blocked or unfilled constrictions out of 49 in our PDMS prototypes is a manageable amount that will not impede the devices from functioning in practical applications. Also hundreds of constrictions could be utilized in complex environments to account for unexpected blockages [250].

The isolated species were removed from the isolation chambers, for further analysis and culturing. We removed the separated CFP P. aeruginosa cells from the food chambers and spread plated them onto LB-agar plates. After overnight incubation, the plates where inspected under a microscope and only cyan colored colonies were observed, further confirming successful separation of an individual species.
Table 1: Separation efficiency data of devices: 49 constrictions with various widths were used to separate a culture of CFP *P. aeruginosa* and m-cherry *E. coli* mixed at a same v/v and concentration ratio (adapted from ref [250]).

<table>
<thead>
<tr>
<th>The width of 950 nm tall constrictions (µm)</th>
<th>Number of constrictions tested</th>
<th>Isolation chambers with single species</th>
<th>Isolation chambers with multiple species</th>
<th>Isolation chambers with no cell growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Multiple lines in constriction</td>
<td>Single line in constriction</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>7</td>
<td>5</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>1.75</td>
<td>7</td>
<td>-</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>4</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>2.5</td>
<td>7</td>
<td>4</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>11</td>
<td>11</td>
<td>7</td>
</tr>
</tbody>
</table>

5.1.2.2. **GFP and M-cherry Escherichia coli Cultures**

The goal of this set of experiments was to confirm that when a single bacterial cell squeezed into a constriction it would prevent other cells, including the cells from the same species from entering. To demonstrate this, GFP and m-cherry *E. coli* strains were cultured, mixed at equal volumes and inoculated into the device. None of the *E. coli* strains were able to get inside of the 700 nm tall constrictions. 950 nm tall, 1.5 µm wide constrictions were sufficient to trap an *E. coli* cell; however, structures narrower than 1.5 µm did not allow any cells to reach the isolation chambers. It is remarkable that only one color of bacteria grew in any given chamber, supporting the hypothesis that the separation is random and that the cells growing inside the isolation chamber are progenies of that single cell in the constriction. Figure 21 depicts the separation of GFP *E. coli* with a 1.5 µm wide and
950 nm tall constriction. It is clearly visible that GFP *E. coli* cells were present along the constriction and in the isolation chamber while m-cherry *E. coli* was only present in the main entrance, and was not able to enter the constriction and the isolation chamber [250].

![Figure 21](image-url)  
**Figure 21:** (A, B) Self sorting of GFP *E. coli* from m-cherry *E. coli* via a 950 nm tall, 1.5 µm wide, 40 µm long constriction. (A) GFP filter, 100X magnification. A single column of GFP-labeled cells is visible. (B) RFP-filter, 100X magnification. No RFP-labeled cells are visible in the constriction or in the isolation chamber (adapted from ref [250]).

**5.1.2.3. A Mixed Culture of Marine Roseobacter sp. and Psychroserpens sp.**

Prior to *in situ* studies, the devices were tested whether they can be used for the separation and cultivation of environmental microorganisms. For this purpose, two previously uncultivated marine species were cultured: *Roseobacter* sp. and *Psychroserpens* sp. Both species were initially checked under a microscope slide to learn more information regarding their morphology. *Roseobacter* sp. is a rod-shaped bacteria and greater in size than *Psychroserpens* sp. As can be seen from Figure 22, *Roseobacter* strain is rod-shaped with an average diameter of 0.9 µm. The shape of
Psychroserpens sp. changes with the age of the culture, with young cultures having rodlike cells that are 2-6 µm long and 0.5-0.6 µm wide, and older cultures of transforming into a coccoid shape with diameters between 0.5-2.0 µm [263].

![Micrographs of (A) Psychroserpens sp. and (B) Roseobacter sp. strains, respectively.](image)

Figure 22: Micrographs of (A) Psychroserpens sp. and (B) Roseobacter sp. strains, respectively.

Even though both species were not labeled with fluorescent protein genes for rapid detection, the presence of both species in the device can be simply distinguished by the differences in their morphology. Figure 23 (A,B) shows an isolation chamber connected to the main entrance with a 3 µm wide constriction. Both species grew in the main entrance of the device, but only Roseobacter sp. was present in the constriction and in the isolation chamber, which could be distinguished by its morphology. The bacteria grown in the isolation chamber was collected and cultured on LB-agar plates, which confirmed that Roseobacter sp. was the only species present in the chamber (Figure 23 C,D).

The images demonstrated that 950 nm tall, 3 µm wide constrictions showed multiple lines of cells; however, narrower constrictions did not show any bacterial growth. This suggests that a wider range of constriction sizes may be needed for separating certain bacterial species in the environment. This observation also raises several questions regarding the behavior of bacteria in confined spaces [250].
These results provided initial understanding that the devices could be used to cultivate environmental species, and that the design is effective for separating previously uncultivated species.

![Image of Roseobacter separation](image)

**Figure 23:** Separation of *Roseobacter* via a 950 nm tall, 3 µm wide constriction. (A-B) 40X and 100X brightfield images from the same section of the device. *Roseobacter* sp. was present both in the main entrance and the isolation chamber whereas *Psychroserpens* sp. existed only in the main entrance. (C) The brightfield image of individual *Roseobacter* sp. cells in LB. The colonies were randomly picked from the plate shown in (D). (D) The photograph of the culture plate containing only *Roseobacter* sp. colonies. The sample was collected from the isolation chamber shown in (A-B) and spreaded onto the LB-agar plate.

5.1.3. Computational Modeling of the Mass Transport in the Device

The computational modeling of the diffusion rate of molecules inside the device is important for validating the effectiveness of the current device design as the chemo-attractant gradient in the isolation chambers needs to be maintained for
several days to attract bacteria toward the constrictions. To investigate this, the model was constructed as one isolation chamber connected to an open reservoir via a constriction. Leucine and serine are the two amino acids used in this study that are found in LB and are known to be as chemo-attractant to bacteria [140, 264]. The reservoir contained only water, which represents the environmental conditions such as the scarcity of nutrients and the effects of bacterial growth and their nutrient consumption in the main entrance. The concentration of leucine and serine in the isolation chamber versus time was separately analyzed, and plotted as seen in Figure 24. The model showed that it takes approximately 7 days for 95% of the leucine, and 6 days for the 95% of the serine to diffuse. This duration is sufficient to enable bacterial cells to grow in the main entrance, move chemotactically to the constrictions and reach the isolation chamber by division.
Figure 24: (A,B) The plots demonstrating the concentration of Leucine (black color) and Serine (blue color) in the isolation chamber versus time, respectively. The amino acids are diffusing from the isolation chamber into the main entrance via a constriction (adapted from ref [250]).

5.2. Aim 2

5.2.1. Nanoporous Membranes on Microfluidic Devices

There are important factors that affect the efficiency of a nanoporous membrane on a microfluidic device: the porosity of the membrane, the sealing methods, and the compatibility of the membrane material to the environment. These considerations were tested using polycarbonate (PC) and Anodisc membranes. Both membranes are hydrophilic which is crucial to maintain the interface between the
device and the environment. The Anodisc used in this study is an unsupported aluminum oxide membrane, 13 mm in diameter with a pore size of 0.02 µm, and the PC membrane is 25 mm in diameter with a pore size of 0.05 µm (Figure 25). Their well-defined pore size is small enough (0.02-0.05 µm) to prevent bacteria from directly entering into the isolation chambers and contaminating the device but also large enough to promote the diffusion of small molecules into the device to cultivate the isolated bacteria. These membranes are also stable at harsh environmental conditions and are non-degradable by bacterial enzymes.

Negative controls were performed with a PC membrane to test the effectiveness of nanoporous membranes in preventing contamination in the isolation chambers (Figure 26). A total of 3 devices were tested, each having a main entrance and 7 isolation chambers. Two out of 3 devices indicated no bacterial growth and the third device had bacterial growth in only 2 isolation chambers. However, this may be explained by improper sealing, as the PC membrane tends to form ruffles on the device surface while sealing. Furthermore, the irregularity in the shape may become more distinct over several days of incubation inside the culture. Therefore, proper
The handling of the membrane is crucial to prevent membrane ruffle formation and the resulting contamination.

![Image](image.png)

**Figure 26:** The brightfield image of a negative control device with a PC membrane on top of all the access holes. Both the isolation chamber and the main entrance of the device did not show any bacterial growth after 2 days of incubation at room temperature in a bacterial culture.

5.2.2. Diffusion Studies

5.2.2.1. Determination of the Diffusion Coefficient

Pyocyanin was chosen as the diffusing species for the diffusion studies, because it represents a standard signaling molecule that is expected to diffuse through the membrane-PDMS interface separating the bacteria from its environment. It is a redox-active, small molecule (210.23 Daltons) secreted by *P. aeruginosa.*
Because it is redox-active, an electrochemical sensor could be used to detect its presence [254].

The diffusion coefficient of pyocyanin in PBS was empirically determined using cyclic voltammetry (Figure 27). Randles-Sevcik equation indicates that the current peak height will increase linearly with the square root of the scan rate in a reversible system. The slope of the resulting line will have a value proportional to the diffusion coefficient of the molecule, which can be seen in Figure 28. The diffusion coefficient for an electroactive species can thus be determined using the Randles-Sevcik equation:

\[ i_p = 0.4463 nF \sqrt{\frac{nFD}{RT}AC\sqrt{\nu}} \]  

(4)

where:  
- \( i_p \) = Peak current (A)  
- \( n \) = The number of electrons transferred (2 for the redox reaction of pyocyanin)  
- \( F \) = Faraday’s Constant  
- \( R \) = Gas constant  
- \( T \) = Temperature (K)  
- \( A \) = Electrode area (cm\(^2\)),  
- \( C \) = Concentration (mol/cm\(^2\))  
- \( \nu \) = Scan rate (V/s)  
- \( D \) = Diffusion coefficient (cm\(^2\)/s)

The diffusion coefficient was found to range between \( 6.10 \times 10^{-9} \) m\(^2\)/s and \( 9.54 \times 10^{-10} \) m\(^2\)/s for anodic and cathodic peak currents, respectively. These values are within the range of diffusion coefficients stated in the literature for molecules with
similar molecular weights [265]. $6.10 \times 10^{-9}$ m$^2$/s was inserted into the COMSOL models as the experimental diffusion coefficient.

Figure 27: Cyclic voltammogram of 10 μM pyocyanin in 10 mM PBS from -0.7 to 0.0 V at a scan rate of 0.5 V/s. Cathodic and anodic peak currents can be obtained using a baseline-subtraction method to determine the maximum currents of those peaks. Note: The current polarity was set to anodic positive on the potentiostat.
Figure 28: Plot of peak current (A) vs. scan rate $^{1/2} (V/s)^{1/2}$ of 10 µM Pyocyanin molecule in 10 mM PBS solution. The diffusion coefficient can be determined from the slope of the resulting lines using the Randles-Sevcik equation.

5.2.2.2. Detection of Pyocyanin Concentration in the Isolation Chamber over Time

The results elucidated that the diffusion rate was faster with no membrane than it did with the PC or Anodisc membranes (Figure 29). With no membrane, the concentration of pyocyanin reached equilibrium at approximately 20 minutes while it took slightly longer than 30 minutes with the Anodisc membrane. Pyocyanin concentration with the PC membrane did not reach equilibrium even after 50 minutes of scanning. It is important to note that the device with the Anodisc membrane demonstrated a faster diffusion rate than with the PC membrane, which was expected due to the greater porosity of the Anodisc membrane (Figure 29 D-F).
The theoretical models were in agreement with the experimental data (Figure 29A-C). The COMSOL model processed that the pyocyanin concentration with a PC membrane would reach equilibrium after 100 minutes. As expected, this is slower than what is observed with the Anodisc membrane.

The empirical diffusion coefficient value used for the COMSOL models has a significant impact on the diffusion rate data. To optimize this, a variation of diffusion coefficients, which were kept within the range of literature values, was tested. The greatest fit to the experimental data was achieved with an optimized diffusion coefficient of $9 \times 10^{-9} \text{ m}^2/\text{s}$ vs. the experimentally determined value of $6.10 \times 10^{-9} \text{ m}^2/\text{s}$ (Figure 30-32).

Another factor that was evaluated theoretically in COMSOL was the effect of 2D vs. 3D modeling in estimating pyocyanin diffusion concentration. The 2D diffusion model led to a significantly slower diffusion rate compared to the 3D models (Figure 30-35). This can be explained by the differences in diffusion in spatial coordinates between 2D and 3D. Furthermore, it is noted that the number of pores designed in the model affects the diffusion rate as increasing the number of membrane pores placed into the model improved the unidirectional behavior of diffusion in the 3D coordinate system (Figures 31-32). However, the number of pores used in the models was kept low due to limitations of computational memory.

The diffusion of essential environmental molecules into the device needs to be rapid and sustained to promote the growth of the isolated bacterial cells. Figure 29 demonstrates that the diffusion of a small molecule from the environment to the isolation chamber takes less than two hours to reach equilibrium. This diffusion rate
is sufficient to allow the devices to be placed \textit{in situ} samples such as the human fecal matter, which requires at least few days for bacteria to grow in the chambers.
Figure 29: Effect of a nanoporous membrane covering the access hole of an isolation chamber in the diffusion of pyocyanin into the device. (A-C) COMSOL model showing the surface concentration of pyocyanin throughout the access hole after 10 minutes (A) without a membrane, (B) with a PC membrane, and (C) with an Anodisc membrane. (D-F) Plots depicting the empirical and computational data of pyocyanin concentration at the bottom of the access hole over time (D) without a membrane, (E) with a PC membrane, (F) with an Anodisc membrane (blue lines represent the computational data; black points represent the empirical data).
Figure 30: Measurement of pyocyanin concentration at the surface of the electrochemical sensor over time as it diffuses from the top of an open well with no membrane (black line). The well was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin. Theoretical models of 2D (gray line), and 3D transport, using either the experimentally determined ($6.10 \times 10^{-9}$ m$^2$/s) (red line) or an optimized diffusion coefficient ($9 \times 10^{-9}$ m$^2$/s) (blue line).
Figure 31: Measuring pyocyanin at the surface of the electrochemical sensor over time as it diffuses through a well that is covered with a polycarbonate membrane (black line). The well was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin on top of the membrane. Theoretical models of 2D (green line), and 3D transport using the experimentally determined \((6.10\times10^{-9} \text{ m}^2/\text{s})\) diffusion coefficient with multiple pores (red line) and with 1 pore (gray line), and 3D transport with an optimized diffusion coefficient \((9\times10^{-9} \text{ m}^2/\text{s})\) (blue line).
Figure 32: Measuring pyocyanin at the surface of the electrochemical sensor over time as it diffuses through a well that is covered with an Anodisc membrane (black line). The well was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin on top of the membrane. Theoretical models of 2D (gray line) and 3D transport using either the experimentally determined (6.10×10⁻⁹ m²/s) with multiple pores (red line) and with 1 pore (green line), and a 3D transport with an optimized diffusion coefficient (9×10⁻⁹ m²/s) (blue line).
Figure 33: A 2D theoretical model of pyocyanin concentration diffusing down through the isolation chamber to the surface of the electrochemical sensor after 10 minutes, using an open well with no membrane. The bottom rectangle represents the well, which was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin (top rectangle). The experimentally determined \((6.10 \times 10^{-9} \text{ m}^2/\text{s})\) diffusion coefficient was used in the model.
Figure 34: A 2D theoretical model of pyocyanin concentration diffusing down through the isolation chamber to the surface of the electrochemical sensor after 10 minutes, using a well that is covered with a polycarbonate membrane. The bottom rectangle represents the well, which was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin (top rectangle). A single pore between the solutions is used to simulate the active transport area of the membrane. The experimentally determined \((6.10\times10^{-9} \text{ m}^2/\text{s})\) diffusion coefficient was used in the simulation.
Figure 35: A 2D COMSOL theoretical model of pyocyanin concentration diffusing down through the isolation chamber to the surface of the electrochemical sensor after 10 minutes, using a well that is covered with an Anodisc membrane. The bottom rectangle represents the well, which was prefilled with 100 µL of 10 mM PBS prior to adding a 50 µL droplet of 10 µM pyocyanin (top rectangle). A single pore between the solutions is used to simulate the active transport area of the membrane. The experimentally determined ($6.10 \times 10^{-9} \text{ m}^2/\text{s}$) diffusion coefficient was used in the model.

5.2.3. Isolation and Cultivation of Species from Human Fecal Samples

Human fecal samples provided a great example to highlight the effectiveness of the device in situ in isolating and cultivating bacteria from patient samples. The taxonomic assignment was done using NCBI Blast database against Greengenes...
database [266, 267]. The taxonomy of species was compared across isolation rounds using Greengenes identification data.

Eight species out of 39 total isolates were isolated in the isolation chamber of 9 microfluidic devices: *Sutterella, Collinsella, Bifidobacterium, Dialister, Enterococcus, Slackia, Dorea formicigenerans, Ruminococcaceae*. These microorganisms are low abundant species in human samples and often cultured only in enriched media. When the frequency of the 8 species isolated in the device was compared with the frequency on the plates, only Plate 3 showed relatively high growth of *Collinsella* and *Bifidobacterium* species where Plate 1 and Plate 2 showed almost no growth of any of the 8 species at that taxonomic level collected with the devices (Table 2). The devices; however, did not exhibit any highly abundant microorganisms, which could be due to the fewer number of devices placed into the fecal matter. As more isolation chambers are deployed *in situ*, the species richness is expected to increase as well.

In addition, the species richness in sub-microfluidic devices was compared with the richness obtained with the conventional plate culturing. Even though the species richness appears lower in the devices than on the plates, when it is scaled with respect to the number of isolates recovered, the fraction is actually very close to the value obtained on Plate 1 and better than the values obtained on Plate 2 and Plate 3 (Table 3).
Table 2: A comparison between the frequency of taxonomic level obtained with the devices to the conventional plates.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Device (Total 39)</th>
<th>Plate 1 (Total 92)</th>
<th>Plate 2 (Total 83)</th>
<th>Plate 3 (Total 85)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sutterella</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>Collinsella</td>
<td>0.13</td>
<td>0.00</td>
<td>0.00</td>
<td>0.26</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>0.31</td>
<td>0.07</td>
<td>0.01</td>
<td>0.25</td>
</tr>
<tr>
<td>Dialister</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>Slackia</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Dorea</td>
<td>0.08</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>formicigenerans</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruminococcaceae</td>
<td>0.08</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 3: Species richness in devices and plates with respect to number of isolates collected.

<table>
<thead>
<tr>
<th></th>
<th>Species Richness</th>
<th>Isolate Count</th>
<th>Richness Scaled to Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Device</td>
<td>8</td>
<td>39</td>
<td>0.2051</td>
</tr>
<tr>
<td>Plate 1</td>
<td>19</td>
<td>92</td>
<td>0.2065</td>
</tr>
<tr>
<td>Plate 2</td>
<td>14</td>
<td>83</td>
<td>0.1687</td>
</tr>
<tr>
<td>Plate 3</td>
<td>10</td>
<td>85</td>
<td>0.1176</td>
</tr>
</tbody>
</table>
5.2.4. Isolation and Cultivation of Species from Aquatic Samples

The water samples from the Charles River were collected both in winter and spring terms from the same location, and the bacterial distribution observed in devices with respect to seasons was compared.

Bacterial species observed varied between devices, and each of which was often dominated by different species. The spatial distribution of bacteria in the environmental sample allowed distinct species to surround the devices and become randomly isolated in each device. Also chemical interactions might be playing a significant role in the isolation and the growth of certain species in one device. To improve the yield, more devices could be deployed into the environment to recover more species.

When species collected from the winter and the summer samples were compared, more species were obtained during the summer season than the winter season (Figure 36). One possible reason for this is that bacteria in cold conditions grow more slowly and often stay dormant, leading to a lower number of species recovered. The isolated species included fresh water and soil microorganisms, and their presence in the river noticeably varied between the summer and the winter. This variation in species distribution in different seasons was also reported by researchers [268]. Furthermore, 2015 winter, when the winter sample was collected, was one of the most severe winters in Boston’s history, and almost all the river was frozen. This might also have an effect on the number of species observed in the winter. Most bacteria collected in the sample were psychrophilic.
Figure 36: (A-B) Taxonomic tree obtained from the Charles River. (A) The winter samples (B) The summer samples.

In addition to the field studies performed in the Charles River, 28 devices were deployed into a lake in Greenland for several weeks. Initial findings elucidated that number of isolated species in the isolation chambers varied from 0 to 5 (Table 4-5). The width of the constrictions did not have a significant effect on the number of species grown in the chambers. Further analysis is being carried out to identify the isolated species.

Table 4: Number of species grown in each isolation chamber of sub-microfluidic devices with respect to the width of 700 nm tall constrictions.

<table>
<thead>
<tr>
<th>The width of 700 nm tall constrictions</th>
<th>Number of species grown in each isolation chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dev. 1</td>
<td>Dev. 2</td>
</tr>
<tr>
<td>1.75</td>
<td>5</td>
</tr>
<tr>
<td>1.25</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>0.75</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5: Number of species grown in each isolation chamber of sub-microfluidic devices with respect to the width of 950 nm tall constrictions.

<table>
<thead>
<tr>
<th>The width of 950 nm tall constrictions</th>
<th>Number of species grown in each chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Device 1</td>
</tr>
<tr>
<td>1.75</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
</tr>
</tbody>
</table>

5.3. **Aim 3**

Bacteria in nature live in confined spaces and porous materials, and are also exposed to constrictions in a variety of man-made settings, ranging from surgical masks to water filters. Bacterial morphology is well understood, however, far less information is available about bacterial stiffness [269].

The use of fluid flow to control bacteria is critical for not only confining and studying single cells, but also for understanding large-scale processes, such as water filtration. Individual cells migrate and move to new locations from biofilm communities in response to physical and chemical stimuli in natural and man-made environments. One example where movement of cells is important is slow sand filtration, which is a cost-effective water treatment process that uses sand grains to filter surface water. The efficacy of the process is acknowledged by World Health Organization [270].
The shortage of clean drinking water is already a global crisis leading to more deaths than war. According to the National Academy of Engineering, providing access to clean water is one of the 14 major grand engineering challenges. In many heavily polluted areas, it is necessary to filter out harmful bacterial pathogens to obtain safe drinking water. Depending on the sand grain dimension and the stacking configuration, the percolated channels of interstitial space can lead to physical straining, in that, cells or multi-cell aggregates larger than the channel dimension are retained while clean water passes through. To evaluate the pragmatic effectiveness of nano-sieves and to optimize their performance, it is necessary to investigate the mechanical behavior of single bacterial cells and the mechanism of how they tunnel through pores and channels. In the following sections the results of this study will be discussed in detail.

5.3.1. Deformation of PDMS devices

PDMS is an elastic polymer, and this characteristic slight deformation when it is attached to glass or exposed to pressure driven flow [247, 271]. While the current protocol of PDMS preparation for most biological studies is of 10:1 base to curing agent ratio, this ratio was modified to 5:1 to minimize possible structural changes of our features [272]. Elastic modulus describes how resistant bacteria are to deformation upon applying force, and is a factor in bacterial behavior in confinements. Bacterial cells become more elastic as the elastic modulus decreases, and a typical bacterial cell has an elastic modulus in the range of 100 – 400 kPa [273].
Since the fluid flow is pressure driven, the pressure drop in the microchannels can be analyzed with the Hagen-Poiseuille equation:

$$\Delta P = \frac{128 \mu L Q}{\pi D_H^4}$$  \hspace{1cm} (5)

where: $\Delta P$ = Pressure drop (Pa)

$\mu$ = Dynamic viscosity (Pa.s)

$L$ = Length of the channel section (m)

$Q$ = Volumetric flow rate ($m^3/s$)

$D_H$ = Hydraulic diameter (m)

Given that the volumetric flow rate is the same in each section of the microchannel and the pressure drop between the inlet and outlet is known, the pressure drop in each section can be computed. The fluid is incompressible and there is no slip boundary condition. The design values provided in the Methodology Section were used, and theoretical modeling elucidated that the vast majority of the pressure drop (99.99%) occurs in the sub-micrometer constriction; therefore the pressure difference between the inlet (known applied pressure) and the outlet (atmospheric pressure) is an excellent approximation of the pressure drop across the length of the constriction (Figure 37 A-F). Bacterial cells therefore directly experience the pressure values applied from the pressure inlet. Importantly, the pressure drop only occurs before reaching the constrictions when the cells start to accumulate, which could alter the pressure head the bacterial cells are exposed to at the entrance of the constriction (Figure 37 F). This variation could be avoided by diluting the bacterial culture so that the cells would not accumulate at the entrance of the constriction. Furthermore, a second cell can be seen entering the confinement
before the first one exits in Figure 45. This is likely due to surface modifications on the PDMS created by the first cell as it moved through the constriction. The cells also continue to accumulate at the entrance while the first cell is in the confinement. This observed phenomenon requires separate additional investigation.

The applied hydrostatic pressure is the best an indicator of external load to promote cellular forward locomotion, rather than an accurate measurement of the pressure acting on individual cells in the channel.

Figure 37: (A-F) The 2D theoretical models of pressure drop in the sub-microfluidic device as 100 mbars of pressure (red color) was applied from each pressure inlet and the outlet was open to atmospheric conditions (blue color). (A) The pressure profile within the whole microfluidic device. (B) The pressure drop within the constriction without any bacteria. (C) The pressure drop profile as one bacterial cell is at the entrance of the constriction. (D) The pressure drop profile as one bacterial cell gets squeezed inside the constriction. (E) The pressure drop profile as two bacterial cells are at the entrance of the constriction. (F) The pressure drop profile as a bacterial cluster accumulates at the entrance of the constriction.
It is remarkable to note that high-pressure values could cause leakage in the device. In this study, pressure could easily build-up around the sub-micron constrictions of the microfluidic device as bacteria accumulate at the entrance of the constrictions and prevent fluid flow. The bonds between the PDMS device and the glass coverslips cannot withstand the high pressure head values and detaches, leading to the failure of the device. A microfluidic device that is permanently bonded to a glass coverslip often withstands up to 4 bars of pressure head [274]. By using a flow controller system, the pressure applied into the system could be modulated. Also, both the bacterial studies and the bead studies were performed at pressure values below 4 bars.

5.3.2. Polystyrene Beads

Fluorescent polystyrene beads that have the similar sizes as bacteria were chosen as a control as they are excellent to gather information about how robust and incompressible structures behave in constrictions under applied pressure.

The pressure inlet channel was easily filled with beads at pressure values as low as 0.1 bars. However, beads started to enter into the 950 nm tall, 5 µm wide constriction at 1.6 bars of applied pressure as can be seen in Figure 38 (A, B) while they entered 950 nm tall, 2.5 µm wide constriction at 2.3 bars (Figure 38 C, D). As the constriction width narrowed down to 1.5 µm, the applied pressure was increased to 3.4 bars to observe a couple of beads in the constriction (Figure 38 E, F). These observations elucidate that it is possible to force incompressible beads into constrictions smaller than their diameter, indicating that the PDMS polymer can be
deformed. As the constriction width narrowed down, significantly higher pressure values were required to force the beads into the constrictions [275].

![Figure 38: Images of 1 µm diameter beads passing through 950 nm tall constrictions with varying widths. (A-B) 20X, brightfield and GFP filter images of beads that entered 5 µm wide constrictions at 1.6 bars of pressure, respectively. (C, D) 40X, brightfield and GFP filter images, respectively. Beads entered 2.5 µm wide constrictions at 2.3 bars of pressure. (E, F) 40X, brightfield and GFP filter images, respectively. Only 1-2 beads were seen inside the entrance of 1.5 µm wide constrictions with 3.4 bars of applied pressure (adapted from ref [275] © 2014 IEEE).](image)

**5.3.3. Bacterial Studies**

There are several factors contributing to the bacterial behavior in constrictions under applied pressure: changes in bacterial cell size due to variations in their growth phase or genetic factors, and the critical size of the constrictions [276, 277]. To abate these variations, all bacterial species were cultured until the stationary phase of growth as the bacteria in stationary phase have gone through most of the variability in length and diameter occurring during exponential phase. To investigate the size of bacteria at stationary phase (after 18 hours of growth), SEM
images of *E. coli* and *P. aeruginosa* were taken (Figure 39-40). It was observed that the diameter of both species was remarkably smaller than expected. One possible explanation for this is that the SEM sample preparation protocol, which was explained in detail in Appendices, involves chemicals that fix and dehydrate the cells, leading to substantial changes in bacterial morphology. Volkmer *et al.* performed an extensive study on how different conditions affect the cellular morphology of *E. coli*, and concluded that while the cellular length varies with the growth phase bacteria are in and the media they grow in, their width is kept approximately 1.26 µm ± 0.16 µm [278].

![Figure 39: (A-B) The SEM images of *E. coli* cells.](image)

![Figure 40: (A-B) The SEM images of *P. aeruginosa* cells.](image)
As the cells crossed the constrictions, it was often observed that bacteria, particularly *P. aeruginosa*, continued to grow after overnight incubation (Figure in Appendices).

### 5.3.3.1. *E. coli*

The behavior of *E. coli* cells was examined by flowing them through both 700 nm and 950 nm tall constrictions that had widths varying from 1.25 to 3.0 µm. As shown in Figures 41 and 42, the cells entered the constrictions, which were wider than 1.5 µm at pressure values lower than 100 mbar. As the width of the constrictions narrowed down below 1.5 µm, the pressure value that the cells need reach the constriction noticeably increased, and varied as the experiment was repeated. The higher average pressure needed for cells to enter into 950 and 700 nm tall constrictions with widths of 1.5 µm or narrower suggests a critical constriction dimensions. The data indicates that *E. coli* are capable of entering constrictions that are narrower than their average diameter as long as they have room to flatten and maintain their cross-sectional area. A similar trend was also observed in trapping bacteria in passive sub-microfluidic devices as discussed in detail in Aim 1, where *E. coli* cells did not enter 950 nm tall constrictions that were narrower than 1.5 µm [250]. Other studies also demonstrated that *E. coli* cells could enter constrictions with lower heights than their diameter only when they could spread out along the width of the constriction [24].

The large fluctuations in the pressures required for entry are likely caused by several factors. One factor is the likely variations in cell size as the small cells continue to enter and pass through the confinement easily, but the cells of average size require a greater external form to be squeezed through. The amount of force needed to deform the
cell and move it into a constriction with features below the critical size varies substantially [255, 275]. A second observation is the effect of surface properties of PDMS on bacterial behavior. Importantly, newly bonded PDMS devices are more hydrophilic and as they become exposed to air they return to their hydrophobic state. Bacteria are known to attach to hydrophobic surfaces more than the hydrophilic surfaces, and this attachment could increase the applied pressure values recorded [279]. The third factor could be the accumulation of bacterial cells at the entrance of the constriction, which causes a rapid pressure drop and leading cells to experience lower pressure values than the applied pressure values as mentioned earlier in this Section. A minimum of three separate experiments was performed for each constriction geometry, unless explicitly noted. One-way analysis of variation (ANOVA) was used to determine the statistical significance of resulting measurements, which will be explained in the following Data Analysis Section.
Figure 41: Applied pressure change needed for *E. coli* cells to enter 950 nm tall constrictions with varying widths. # indicates sample size is 2 for that constriction. Error bars indicate standard deviation.
Figure 42: Applied pressure change needed for *E. coli* cells to enter 700 nm tall constrictions with varying widths. As the constriction width reaches 1.5 µm, the force needed to move cells into the constriction increases significantly. Error bars indicate standard deviation.

5.3.3.2. *P. aeruginosa*

In Aim 1, it is elucidated that when there is no fluid flow, *P. aeruginosa* cells entered both 700 nm and 950 nm tall constrictions, though 700 nm tall constrictions were more effective for trapping the cells [250]. Here in Aim 2, *P. aeruginosa* cells entered both 950 nm and 700 nm tall constrictions as the cells were flowed using increasing pressure values applied to the bulk fluid. There was no pattern in the recorded pressure head values as the cells were passing through 950 nm constrictions, which is indicative of the width of the constriction (Figure 43). The
required pressure values to observe at least one cell inside the constriction were often less than 10 mbar. The pressure profile of the bacteria significantly changed with the 700 nm tall constrictions. Figure 44 depicts that as the width of the constriction narrowed down from 2 µm to 1 µm, the pressure applied increased, and significantly higher pressures were required for *P. aeruginosa* cells to enter into constrictions with widths below 1.5 µm, which was also validated with ANOVA test.

![Graph showing applied pressure change needed for *P. aeruginosa* cells to enter 950 nm tall constrictions with varying widths.](image)

**Figure 43:** Applied pressure change needed for *P. aeruginosa* cells to enter 950 nm tall constrictions with varying widths.
Figure 44: Applied pressure change needed for *P. aeruginosa* cells to enter 700 nm tall constrictions with varying widths. * indicates $P < 0.05$ from ANOVA test. # indicates sample size is 2 for that constriction.

### 5.3.3.3. Data Analysis

One-way Analysis of Variance (ANOVA) was performed at 95% confidence level for both *P. aeruginosa* and *E. coli* in 700 nm and 950 nm tall constrictions using Minitab 16 Statistical Analysis program to determine whether the pressure values recorded are significantly different from one another. For *P. aeruginosa*, $P$ values obtained from 700 nm and 950 nm tall constrictions are 0.032 and 0.776, respectively. This indicates that for 700 nm tall constrictions, the mean pressure values of 1 μm wide constriction differs significantly, whereas for the 950 nm tall
constrictions we find no statistical difference between the mean pressures. For *E. coli*, P values obtained from 700 nm and 950 nm tall constrictions are 0.505 and 0.207, respectively. Large P values for the *E. coli* experiments are likely the result of small and unbalanced sample sizes used for the analysis, and would become significant if very large numbers of experiments were performed.

When the behavior of *E. coli* and *P. aeruginosa* in both 700 nm and 950 nm tall constrictions was compared, it was observed that the 700 nm tall constrictions were more restrictive for both species to pass through (Figure 45). *E. coli* required higher pressure values as the width of the constriction reached 1.5 µm, while *P. aeruginosa* required higher pressures to enter constrictions with widths narrower than 1.5 µm (significant increase for 1 µm width). 1 µm diameter beads need very high pressure values to enter 950 nm tall constrictions. In one such example, due to their rigid structure the beads could only enter 2.5 µm wide constriction at 2.3 bars of pressure [275]. These results clearly highlight the deformation in bacterial shape in constrictions, which is critical in understanding the effectiveness of the water filtration processes.
Figure 45: Images of *E. coli* and *P. aeruginosa* passing through the constrictions. (A) Brightfield and (B) RFP filtered images of *E. coli* entering 950 nm tall, 1.5 µm wide constriction at 600 mbar of applied pressure. (C) Brightfield and (D) GFP filtered images of *P. aeruginosa* passing through 700 nm tall, 1.5 µm wide constriction at 10 mbar of applied pressure.

5.3.3.4. Other Microorganisms

In addition to *E. coli* and *P. aeruginosa*, initial tests were performed with *Staphylococcus aureus* and *Psychroserpens* sp. species, which are both non-motile. The preliminary findings revealed that motility may be a critical factor for bacteria to enter and pass through constrictions. Both *S. aureus* and *Psychroserpens* sp. progressed
toward the constrictions at a slower rate, and instead of entering constrictions, they often accumulated at the entrance of the constrictions (Figures 46 and 47). *S. aureus* are spherical in shape with a diameter between 0.5-1.5 µm. The shape of *Psychroserpens* sp. changes with the age of the bacteria from rodlike shape to a coccoid shape as mentioned in Aim1. When compared to *E. coli* and *P. aeruginosa*, it is remarkable to note that *S. aureus* and *Psychroserpens* sp. require both 3 to 5 times more time to reach the constrictions and greater applied pressure head for entry. Further investigation is necessary to understand the cause of this behavior, as the results suggest a complex interplay between shape and surface interactions.

**Figure 46**: (A-C) Brightfield images of *S. aureus* at a 700 nm tall, 2 µm wide constriction entrance after varying applied pressure. Though the applied pressure was increased, the cells could not enter the constriction and accumulated at the entrance of the constriction. (A) 50 mbar. (B) 75 mbar. (C) 100 mbar. Scale bar: 50 µm.

**Figure 47**: (A-C) Brightfield images of *Psychroserpens* sp. at an 870 nm tall, 1.15 µm wide constriction entrance after different applied pressure values. The constriction was narrow enough to prevent cells from entering, resulting in accumulation of cells at the entrance of the constriction. (A) 100 mbar. (B) 300 mbar. (C) 500 mbar. Scale bar: 50 µm.
5.4. **Aim 4**

In addition to their use in self-sorting bacterial species, sub-micron constrictions also enable diffusion of chemicals from one chamber to the other at a certain rate. This property was previously shown in Aim 1 and Aim 2 in terms of nutrient diffusion, and could also be applied for delivering antibiotics to bacteria and observing bacterial response under microscope in real time. For this purpose, sub-microfluidic devices were coupled with Surface Plasmon Resonance imaging (SPRi) instrument to study antibiotic susceptibility of bacteria. While bacteria were exposed to antibiotics, SPRi system could monitor the changes in bacteria in its 1 cm$^2$ field of view [280]. Bright images indicate bacterial growth while the darker images show no growth in SPRi system.

Microfluidic devices were initially only monitored with an optical microscope to observe the effect of antibiotics on *S. aureus*. As can be seen from Figure 48, the exposure of bacterial cells to the antibiotics led to changes in their cellular morphology, making cells swollen. These preliminary results confirmed that the device is suitable for performing antibiotic susceptibility tests.
Figure 48: (A-B) 40X and 100X magnified brightfield images of one part of sub-microfluidic device, showing the morphological changes in \textit{S. aureus} as the cells were exposed to penicillin/streptomycin, respectively. The cells became swollen upon contact with the antibiotic.

Next, the effect of antibiotic concentrations on bacterial behavior was tested by designing a microsystem consisting of a main chamber connected to two microchambers via constrictions made with correctional fluid (17 µm in thickness). Hence this first set of devices is larger in dimensions than the ones microfabricated in cleanrooms. The main channel was initially filled with $5.4 \times 10^6$ cells/mL of \textit{S. aureus}. One microchamber was filled with 200X diluted penicillin/streptomycin in LB while the other microchamber was filled with 1000X diluted penicillin/streptomycin in LB. Bacterial growth was observed continuously as antibiotics diluted 1000X and 200X in LB diffused from small microchambers to the main channel via the constrictions. SPRi difference images after 21 hours of bacterial growth demonstrated that the area surrounding the microchannel with 200X diluted antibiotic was darker compared to the rest of the chamber. In contrast, the region near the 1000X diluted antibiotic chamber is brighter than average (Figure 49). After 21 hours of incubation the nutrients in the main chamber were consumed, keeping the bacteria close to the constrictions though there were fewer cells around
the 200X constriction. This suggests that bacteria are willing to tolerate low levels of antibiotics (1000X) to be closer to a fresh food source [280].

Figure 49: (Top) SPRi difference image 21 hours after the start of the experiment. Lower brightness intensity indicates less biomass accumulation. (Bottom A, B) Brightfield optical images of the regions shown at the top image. In these images, a darker color represents a greater biomass density (adapted from [280]).

Next, in order to permanently bond sub-micron- to nano-sized structures to the gold surface, the surface of the PDMS devices were functionalized with 5mM and 25mM MPTMS solutions. Preliminary studies did not show any specific changes in the bonding efficiency with respect to the varying concentration of MPTMS. As seen in Figure 50, adhesion forces could be strong enough to cause
collapsing in constrictions. Further investigation is necessary to understand this reasons for this interaction.

Figure 50: (A-B) Optical microscope images of bonded PDMS-gold surface devices with 950 nm tall constrictions. The gold surface was functionalized with 25mM MPTMS for 3 hours. (A) 1.5 µm wide functional constriction. (B) 2.5 µm wide partially collapsed constriction.
6.0 Conclusions and Recommendations

In the first aim of this dissertation, a novel sub-microfluidic device was designed, fabricated, and tested as a proof-of-concept with model microorganisms. Different designs and microfabrication techniques were applied to obtain the optimum device to self-sort different bacterial cells. The results showed successful isolation of *P. aeruginosa* from *E. coli*, and a single species was observed in 22 of the 49 isolation chambers tested (45% success rate).

In addition to phenotypic variations in species, physiological aspects such as motility, biofilm formation and chemical production seem to facilitate self-sorting of microorganisms as well. The proposed method can be designed and adapted to minimize these differences by changing nutrient composition in the food chamber and the environmental conditions in the immediate vicinity of the constriction entrance. The devices offer random separation of microorganisms that as one cell gets trapped at the constriction none of the cells could reach to the isolation chambers, which is validated by separating two *E. coli* strains that were labeled with different fluorescent protein genes. Furthermore, initial studies with the two previously uncultivated species were performed, confirming the efficacy of the devices to isolate and grow environmental species.

The second aim focused on creating a prototype sub-microfluidic device to enable *in situ* isolation and cultivation of species. For this purpose, optimizations conducted in the device setup where a polycarbonate or an Anodisc membrane was placed on top of the access holes of the isolation chambers to enable isolated species to grow inside the device. While both membranes are compatible with the PDMS
and the bacteria, there are some challenges in incorporating them onto the PDMS surface. The PC membrane tends to form ruffles on the device surface during the sealing process. This unevenness may become more pronounced over several days of incubation. Anodisc membranes are more porous than PC membranes, but very brittle and crack easily during the sealing step, making them difficult to use in many practical settings. Testing different designs, and different sealing methods are recommended to avoid the cracking and possible contamination issues over long incubation times. Both empirical and computational diffusion rate studies through membranes validated that diffusion rate is sufficient to allow the devices to be placed *in situ*.

*In situ* studies were conducted with human fecal samples, and the aquatic samples, and very promising results were obtained in terms of species isolation and distribution. Deploying more devices into the samples would further enhance the yield. It is important to note that the current device with the long main entrance channel design promotes the separation of motile species more than the non-motile species. Modification in the device design would be highly recommended to allow both non-motile and motile cells to reach the constrictions at the same rate and provide a high-throughput isolation and cultivation. Removing the main entrance channel, in particular, would possibly solve this bias (Figure 51).
Figure 51: (A-B) New platforms to enable the isolation of both motile and non-motile species from the environment. (A) A glass device (B) A high throughput Si-wafer that could contain 384 devices.

The device design shown in Figure 51 (B) could be fabricated in two steps. First, a 6 or 8-inch Si wafer could be partially etched with Deep Reactive Ion Etching (DRIE) until 25-60 µm of constriction length. In the second step, the wafer could be etched thorough to create the diffusion wells (approximately 1 mm in
width) and the constrictions. The width of the constrictions can be kept between 500 nm and 1500 nm, further be reduced by oxidation.

In the third aim, the bacterial behavior in constrictions was investigated under applied pressure. Cell locomotion remains unchanged until an external hydrostatic pressure exceeds a critical threshold, which is related to the ratio of cell dimension to channel cross-section, and likely the ratio of elastic moduli of the cell’s glycoprotein shell and the PDMS wall. This work has significant impact in understanding how physically strained bacteria cells or aggregates pass through a filtrating column.

The experimental setup establishes a method for observing and studying bacterial behavior when they are exposed to external pressure in narrow pores. Different pressure profiles were observed with species. It is recommended to examine multiple microorganisms, the pathogens found in drinking water in particular, to construct a detailed pressure profile for microorganisms, which will improve the separation applications. Another future direction for this study could be examining how mechanical deformation in cellular morphology would affect the uptake of molecules by bacteria from the environment. A similar study was performed on mammalian cells and shown that deformed mammalian cell membranes induce pore formation facilitating the uptake of biomolecules, such as DNA [281]. This study could provide a new approach to transform bacterial cells.

Finally, in the fourth aim, proof-of-concept studies were conducted with *S. aureus* to demonstrate that these versatile devices could also be used to determine antibiotic susceptibility of bacteria. Coupling sub-microfluidic devices with SPRi
systems will provide a dynamic system where the cells and chemical agents can be flowed and monitored continuously in a large field of view. Future work is recommended to investigate the vulnerability of bacterial biofilms by exposing them to varying concentrations of chemicals, and to improve the protocol for bonding PDMS to gold surfaces to avoid channel collapses.
7.0 Nomenclature

2a = Punch spacing (µm)

γ = Work of adhesion (mJ/m²)

E’ = Plain-strain modulus (MPa)

E = Young’s modulus (MPa)

h = Punch height (µm)

D = Dosage (µC/cm²)

A= Total area (cm²)

t = Time (seconds)

I = Current (A)

i_p = Peak current (A)

n = The number of electrons transferred

F = Faraday’s Constant

R = Gas constant

T = Temperature (K)

C = Concentration (mol/cm²)

v = Scan rate (V/s)

D = Diffusion coefficient (cm²/s)

ΔP = Pressure drop (Pa)

µ = Dynamic viscosity (Pa·s)

L = Length of the channel section (m)

Q = Volumetric flow rate (m³/s)

D_H = Hydraulic diameter (m)
8.0 References


143


Figure: The chromatogram of an isolate obtained by using a forward primer in Sanger sequencing.
SEM protocol for preparing bacterial samples

Fixation of the specimen:

- Initially, bacteria were cultured in 6 mL LB for 18 hours. The 12 mm diameter circular coverslips (Fisher Scientific) were cleaned with acetone, treated with a droplet of 0.1% Poly-L-Lysine (w/v) in water for 5 minutes, and rinsed with DI water. A droplet of bacterial solution was placed on a wet coverslip and waited for 5 minutes.

- For primary fixation, the bacterial culture on a coverslip were placed into 10% glutaraldehyde, 0.4 M Na cacodylate and DI water buffer solution at pH 7.2 for 30 minutes.

- The sample was washed three times with 0.1M Na cacodylate buffer at pH 7.2 for 5 minutes.

- The sample was post-fixed with 1.0% osmium tetroxide in 0.1 M Na cacodylate buffer at pH 7.2 for 30 minutes.

- The sample was rinsed again three times with 0.1 M Na cacodylate buffer at pH 7.2 for 5 minutes.

- Samples were dehydrated by treating them in ethanol solutions with various concentrations at room temperature to replace water molecules with ethanol in the specimen:
  - 30% ethanol solution for 8 minutes
  - 50% ethanol solution for 8 minutes
  - 70% ethanol solution for 8 minutes
  - 85% ethanol solution for 8 minutes
- 95% ethanol solution for 8 minutes
- 100% ethanol solution for 30 minutes
- The sample was washed with hexamethyldisilazane (HMDS) three times for 10 minutes and then let it dry prior to sputter coating with 5 nm of Pt.

**Viability Studies**

Figure: (A-D) A viability test conducted on *P. aeruginosa* cells by incubating the device overnight at 37 °C after applying pressure. (A, B) Cells entered 700 nm tall, 1.5µm wide constriction at 10 mbar of pressure. (B) The brightfield image of the device after overnight incubation. (C, D) Cells entered 700 nm tall, 2 µm wide constriction at 10 mbar of pressure. (B) The brightfield image of the device after overnight incubation.