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Master of Engineering
Project Report:

First Steps towards Parameter Optimization of Bioelectrochemical Systems using a Microsystems Engineering Approach

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ABSTRACT
There is a growing interest in exploiting bioelectrochemical systems (BESs), such as microbial fuel cells, as an alternative energy source for sustainable living. Certain species of microorganisms, such as Pseudomonas aeruginosa 14 (PA14) wild type, produce electron carriers, Phenazines, which transfers electrons to the anode in the system and produce digital output signal. The electric current generation of BESs is influenced by many biophysical and biochemical parameters in the system, such as glucose level, cell culture community, cell density, PH, and oxygen level. The existing MFCs are at macroscale, and not suitable for parameter optimization; and as a result they are not yet cost effective. Here, we present our first effort in parameter optimization of BESs using a microfluidic device. Microfluidic device affords us an ability to quickly define a physical and chemical environment for PA14, and its compatibility with microscope allows us a real time observation of the responses. We will present experimental results on the roles of carbon sources in PA14 motility and promising results of Phenazines being a chemoattractant to PA14. We will discuss the relation between PA14 motility and biofilm formation, and subsequently electric current generation.

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BACKGROUND

1.1. Bioelectrochemical Systems: Microbial fuel cells

The BES of interest is the mediator-less microbial fuel cells (MFCs) with the setup as shown in Figure 1 [1,2]. The setup consists of an anode and a cathode chambers separated by a membrane that allows cations to pass through. In the anode chamber, electrochemically active microorganisms such as P. Aeruginosa consume carbon source, such as glucose, in anaerobic conditions and produce electrons and protons. This bacterium is able to secrete electron carriers, Phenazines, which transfer electrons directly onto the anode without the need of any chemical mediators which are usually very expensive. Electrons travel through the external electric circuit into the cathode chamber, while the protons are diffused through the cation membrane. Electrons and protons react at the cathode where they combine with oxygen to form water. Furthermore, since mediator-less MFCs function in wastewater, there is a huge potential to harvest the electricity produced by microbial fuel cells as an alternative power source in wastewater treatment plants. Its ecological and environmental benefits make it a very promising venue for further research.

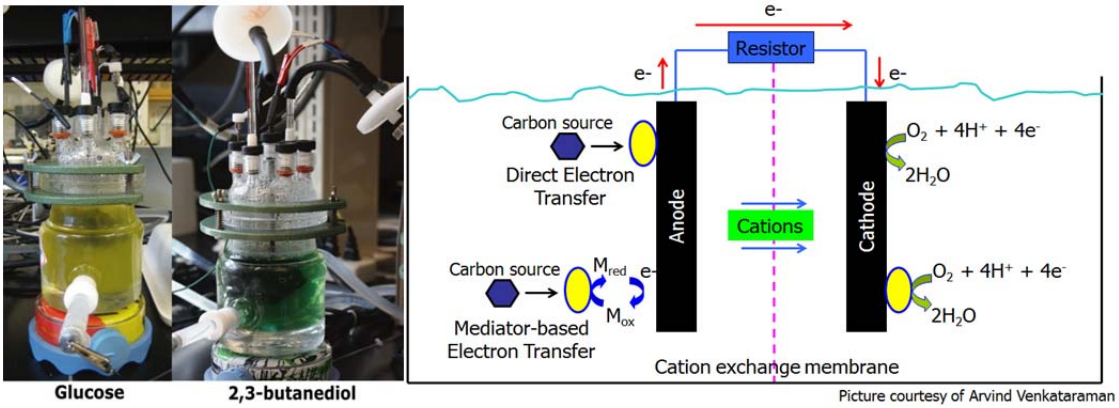


Figure 1: Mediator-less microbial fuel cells in action. [1,2]

(a) Conventional experimental setup; (b) Schematic of a microbial fuel cell system.

2. RESEARCH INTEREST AND MOTIVATION

Despite the largely studied field, the exact mechanism by which electrons are transferred from the Phenazines onto the anode is still unknown. Initial literature review indicates that this is largely due to BES experiments being more frequently conducted in macroscale and there are few studies that focus on the microscale. Macroscale experiments are very useful in determining overall performance of a fuel cell systems and usually requires a bulky setup that takes some time (a few days to a week) to collect a single data point. Within this framework, interest is taken to determine performance factors of a BES that may be worth investigating in microscale.

3. LITERATURE REVIEW

3.1. Previous studies of BES involving P. Aeruginosa

An example of a macroscale experiment involving BES is the studies by Venkataraman et. al. which investigated performance of MFCs using mixed bacterial cultures. A summary of his studies relevant to this project pertains to the performance measures of the MFCs when operated using P. Aeruginosa is shown in Table 1. The study indicated that using different carbon sources, specifically 2,3-butanediol and glucose, as the organic material input in MFCs yield different observed data. When using 2,3-butanediol as a carbon source, current densities observed is higher at 5.2 $\mu A/cm^2$ than at 3.2 $\mu A/cm^2$ with glucose. Production of Phenazines by the bacteria was three times higher using 2,3-Butanediol than glucose and thicker biofilm was observed in the 2,3-butanediol setup than that in glucose.

Table 1: Different carbon sources affects production of current densities in MFCs, phenazines and biofilm by Pseudomonas aeruginosa. [1,2]

Carbon Source	Current densities ($\mu A/cm^2$)	Phenazine concentration ($\mu g/ml$)	Biofilm formed
2,3-Butanediol	5.2	20	More
Glucose	3.2	6.4	Less

From the study by Venkataraman et. al., one could make some inferences on the microscale factors that might influence the overall performance of the system if they were to be adjusted. Microscale factors that may affect electron transfers in MFCs include: (1) Motility of PA14WT in different carbon sources in relation to biofilm formation and (2) Chemotactic effects of Phenazines on PA14WT. Investigating these factors involves creating environments in which bacterial behavior and chemical fluid flow can be easily monitored and controlled respectively. This could be a challenging task to do in macroscale experiments. As such, this is where microsystems engineering could provide a solution.

4. Microfluidic Device

The microsystems engineering approach that could serve the purposes of this study is the microfluidic device. An example of a microfluidic device is shown in Figure 2 with its size being comparable to a penny. This device controls and manipulates fluids at microscale and it is of comparable size to bacteria or cells being studied. In addition, it also provides a controlled environment and has a high throughput due to short experimental time scale as compared to the conventional biological experimental methods.

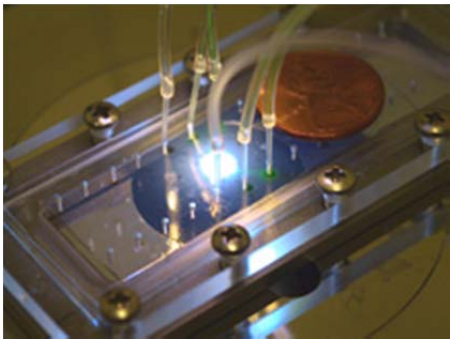


Figure 2: Size of microfluidic device is comparable to a penny

4.1. Hydrogel-based microfluidic device

The microfluidic device to be used for the experiments in this project is the hydrogel-based microfluidic device developed by the Wu Lab in Cornell University. The schematic of the device is shown in Figure 3. It is a 3-channel device that exploits the microscale nature of diffusion to provide controlled microenvironment for bacteria or cells to be studied. It has a channel width of 400µm and a channel height of 170µm. The distance between each channel is 250µm. The device is constructed out of agarose gel which is easy to make and disposable. The inlet and outlet holes allow control of fluid flow within the device.

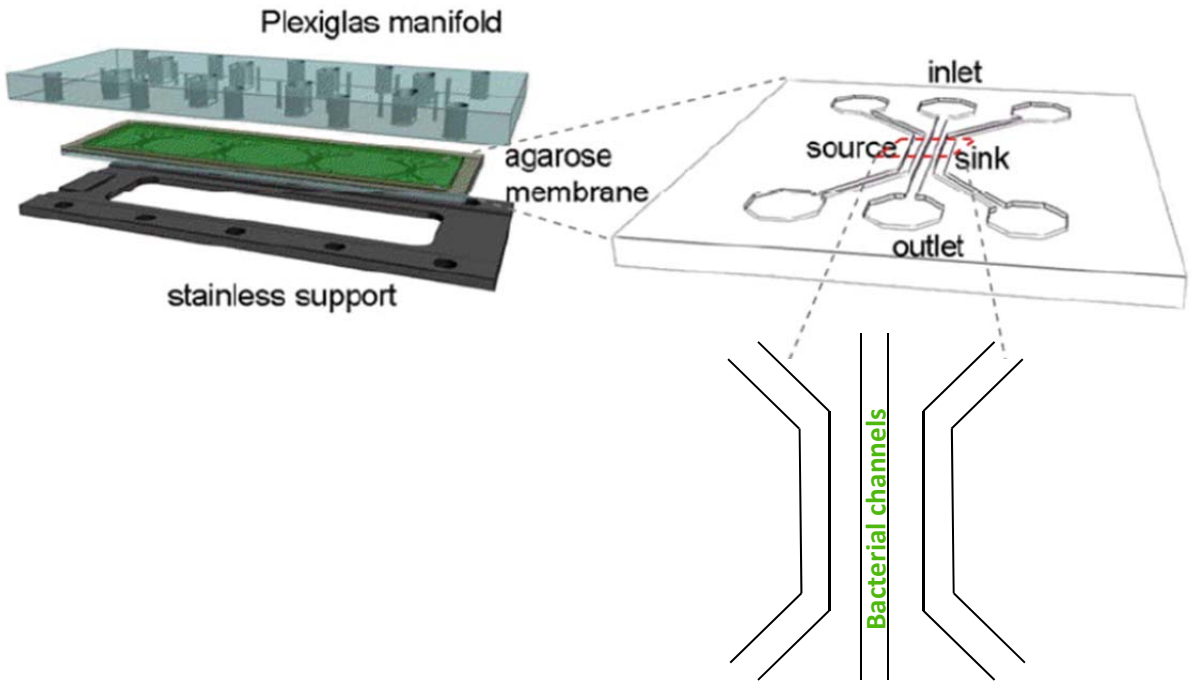


Figure 3: Microfluidic device for environmental control.

4.2. Chemical Gradient Generation

In addition to its capability to manipulate fluid flow at microscale, the device is also capable of generating a chemical gradient across the channels as shown in Figure 4. When the desired chemical is pumped into the source channel while another neutral chemical is pumped through the sink channel, the particles from each channel diffuse through the agarose gel into the center channel, forming a chemical gradient across the channels.

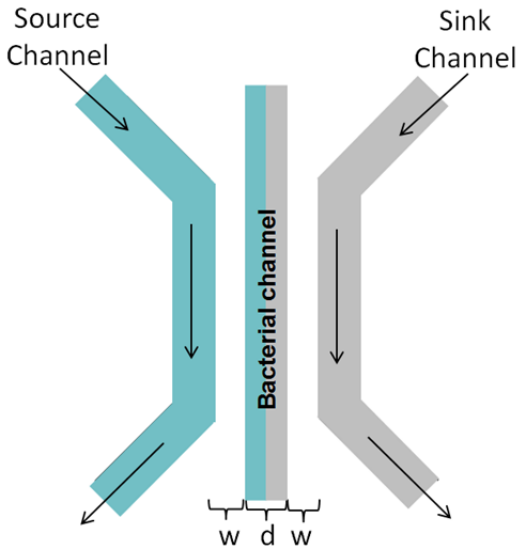


Figure 4: Schematic of chemical gradient generation across the microfluidic channel

The gradient establishing time, *t*, is defined as:

$$t = \frac{l^2}{2D}$$

where *l* is the distance travelled by the particles (microns) and is defined by:

$$l = d + 2w$$

D is the diffusion coefficient, *d* is the channel width and *w* is the distance between channels.

This function enables users to conduct chemotaxis experiments in real time with the ability adjust fluid flow characteristics.

5. Motility of P. Aeruginosa Under Different Carbon Sources

5.1. Pseudomonas Aeruginosa 14 Wild Type (PA14WT)

Pseudomonas Aeruginosa 14 Wild Type (PA14WT), shown in Figure 4a, is a pathogenic bacterium that disintegrates organic waste materials for food. It has dimensions of 5-10μm in length and 0.5-1μm in width. It is a single-cell organism with its swimming motility dictated by a single flagella and its near-surface motility by pili[3]. PA14 is one of the many electrochemically active microorganisms that secrete electron carriers named Phenazines. Relating to previous study by Venkataraman et. al., the motility of PA14WT in different carbon sources at different concentration is the focus of this experiment.

5.2. Experimental Methods

To investigate the motility behavior of PA14WT, we first prepared the bacterium to be experimented. PA14WT is inoculated from the LB agar plate into a test tube with 5ml LB stock solution and left overnight to grow in an incubator. 12-16 hours later, 200μl of the bacteria stock is transferred onto 5ml of 2,3-butanediol and 5ml of glucose solutions (diluted with AB media at 0, 15, 30, 60 and 120 mM concentrations). The tubes are left overnight for about 16-24 hours. The new liquid cultures are sampled in a 200μl 96 well plate in triplicate to figure out their OD and then diluted to 0.04 OD in 1ml tubes with their respective growth media. The bacteria are left aside to settle for about five to ten minutes.

Meanwhile, the hydrogel-based microfluidic device, which had been constructed the day before is prepared for experiments. The channels need to be flushed with the respective growth media so that when the bacteria are loaded into the channels, they will not be too shocked with the sudden change of environment. The bacteria are then loaded into one of the center channel of the device with the appropriate carbon source concentration and then imaged at the microscope at 40x magnification. A 500-frame movie with a frame rate of 1/30 s is recorded. The bacteria-loading and microscope imaging procedure is repeated with the rest of the bacteria grown in different carbon sources at different concentrations. It is important to do the bacteria-loading and microscope imagine step one at a time for each carbon source of a particular concentration so that the bacteria will not slow down waiting too long in the channels. As a side note, the chemotaxi function of the device is not being used at this point of the experiment.

5.3. Results and Analysis

5.3.1.2D Tracking Program (Wu Lab)

The 500-frame movies are analyzed using a 2D tracking program developed by the Wu Lab. A schematic of how the program is shown in Figure 5. The tracking program computes the average velocity of the bacteria in the movies by frame-by-frame image comparison. The contrasting pixels in the frames is given numerical values according to the general contrasts of the image, with black dots (lower values) being regarded as bacteria.

If the black dot moves in the frame, the program will then keep track of these “moving” pixels as x-y coordinates within the movie frame as shown in the “Living, motile bacteria” row in Figure 5. Keeping these x-y values over the course of the movie yields a bacterial trajectory over the whole 500-frame timescale. On the other hand, if the black dot does not move, or remain stationary, in the next image frame as shown in the “Dead Bacteria” row in Figure 5, the black dot is considered a dead bacteria and tracking for that particular dot is discontinued. This discontinuation of tracking also applies to actively swimming bacteria that may swim in and out of focus within the viewing frame of the movie. Thus, even though it may be the same bacteria,

if at some point in time, the bacteria swam out of focus and then reappear, a new set of tracks will begin for that bacteria. The average velocity calculations, however, will still be accurate despite the inconsistency in tracking.

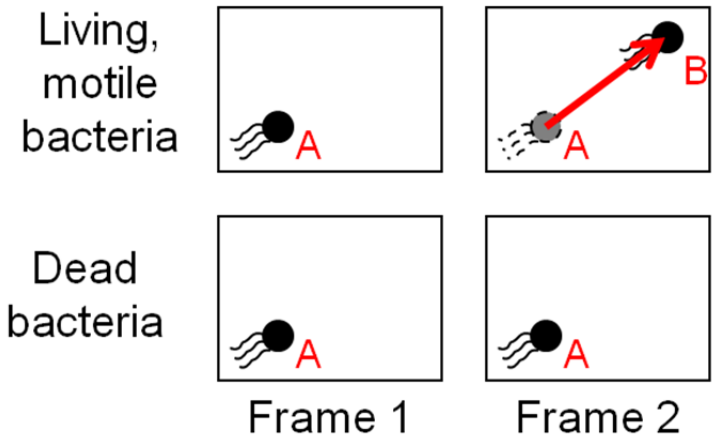


Figure 5: Schematic of 2D Tracking Program. Program only computes average velocity from living, motile bacteria and leave out dead, unmoving bacteria in the movie frames.

5.3.2.Run-and-tumble swimming motility

Initial findings in the motility experiments indicate that PA14WT follows a run-and-tumble motility like the more frequently studied Escherichia Coli as shown in Figure 4. The bacteria trajectory in Figure 4(c) shows distributed, random swimming motility of PA14WT with no specific orientation. A blow-up section of that trajectory shows a run-and-tumble swimming motility of the bacteria. Not all the bacteria project this motility but rather they slow down when they encounter other bacteria in the channel and adjust their trajectories accordingly, forming a slowed down trajectory before proceeding to swim as per normal.

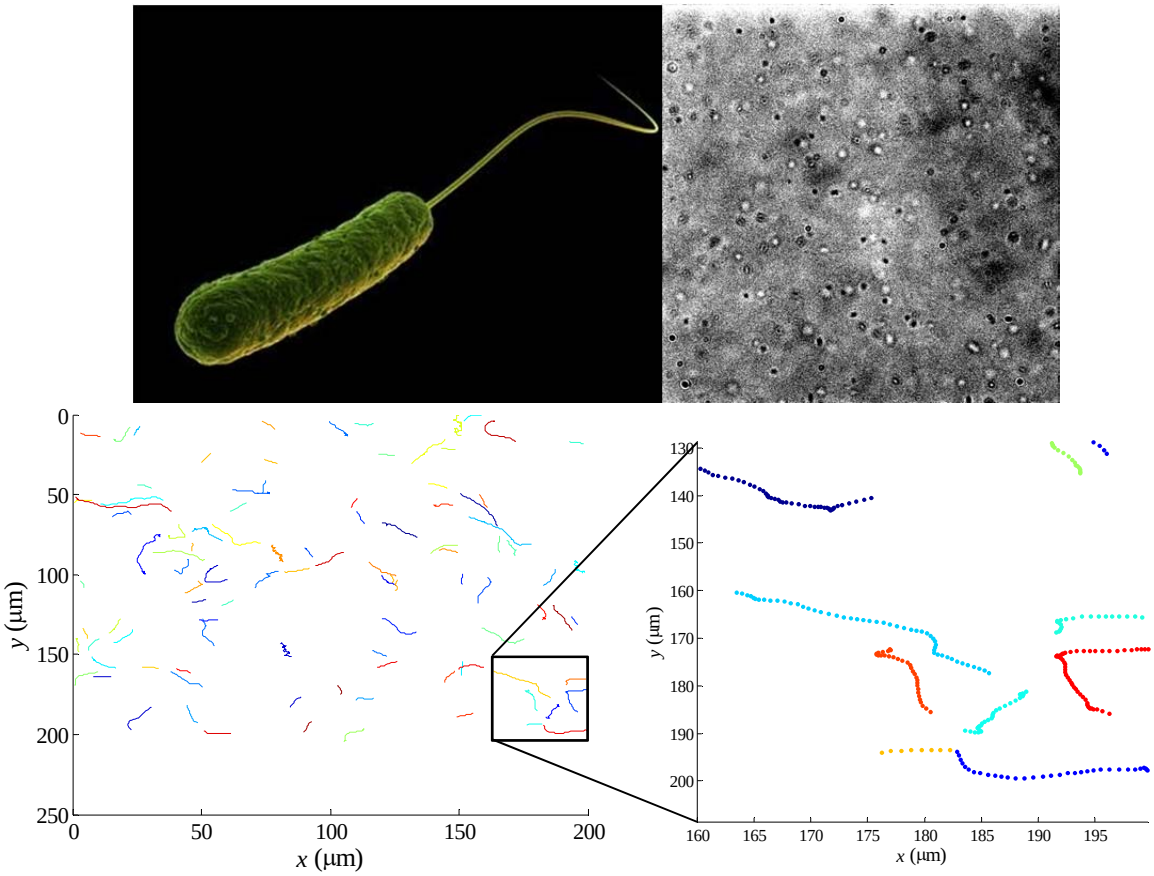


Figure 6: PA 14 follows a run and tumble motility, a similar way to Escherichia coli. (a) SEM image of PA14; (b) an original image of the PA14; (c) Trajectories (A composite trajectories with a blow up of one trajectory).

5.3.3.Effects of carbon sources on motility

The computed average velocity of PA14WT is summarized and compare in Figure 7. The data shows a general upward trend of increasing average velocity (μm/s) with increasing carbon source concentration but slowly flattens out or decrease as the carbon source concentration is much higher. In general, however, the average velocity of bacteria is significantly higher than that in 0mM carbon source concentration (no carbon source available). This indicates that carbon source enhances the motility of PA14WT, with glucose being a more potent carbon source than 2,3-butanediol as the average velocities of the bacteria is always higher in glucose.

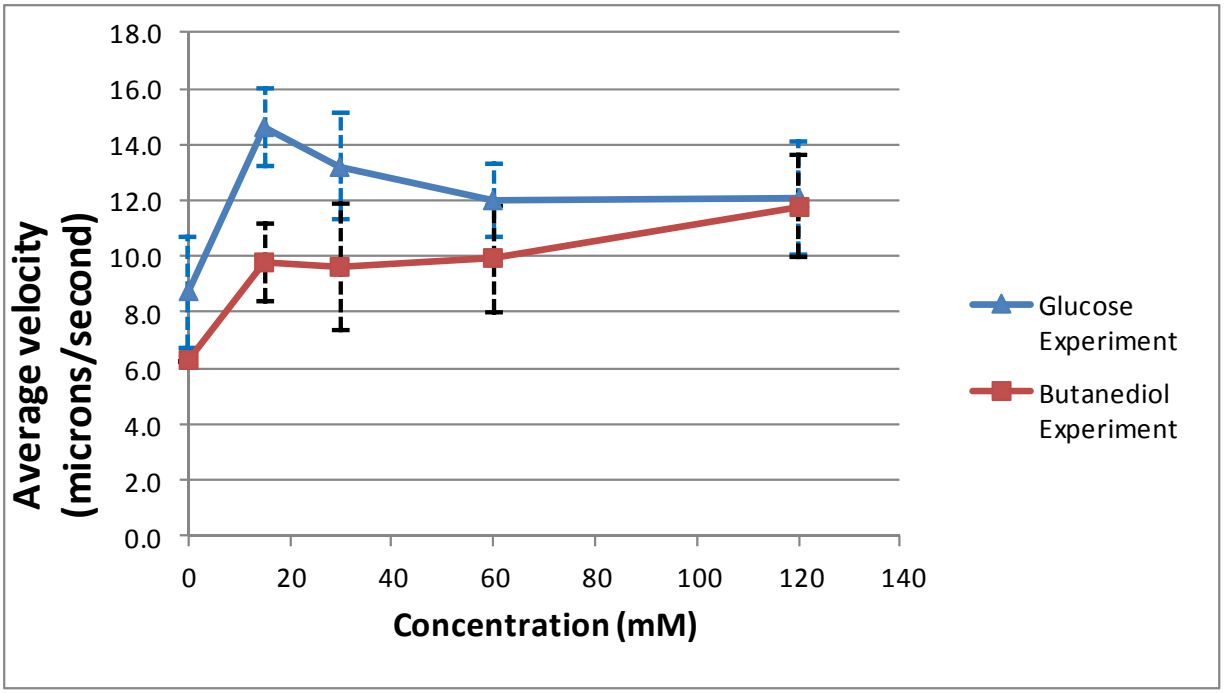


Figure 7: Carbon sources enhances the motility of P. Aeruginosa with glucose being the more potent carbon source.

Relating this to the study by Venkataraman et. al., since more biofilm is formed when the MFC runs on 2,3-butanediol and that bacteria moves slower in 2,3-butanediol than in glucose, there may be a link between the motility of bacteria with biofilm formation. This raises the question if slow-moving bacteria contribute a higher biofilm formation and how, if it does, can one manipulate the bacteria's motility to our advantage in operating BESs and other medical or health applications, for e.g. tumor cells.

6. Are Phenazines Chemoattractant to P. Aeruginosa?

6.1. Phenazines

After characterizing motility of PA14WT in different carbon sources, the next step is to figure out if Phenazines are chemoattractant to the bacteria. Phenazines are compounds secreted by P. Aeruginosa. Being electron carriers, Phenazines facilitate electron transfers onto the anode of MFC. Since the mechanism of electron transfer is still not fully understood, this experiment aims to investigate the chemotactic effects of four types of Phenazines at different concentrations on PA14WT. If Phenazines turn out to be a chemoattractant, it will open doors to the understanding of the electron transfer mechanisms in BESs and biofilm formation. There are four types of Phenazines we will be experimenting on. They are shown in Figure 8.

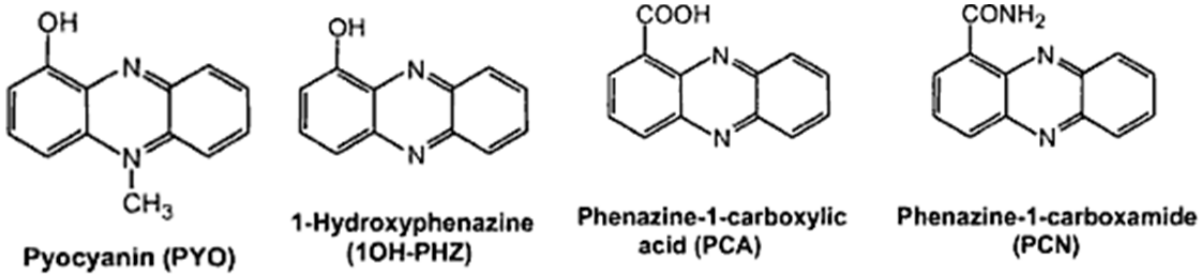


Figure 8: Phenazines secreted by P. Aeruginosa that will be tested for chemotactic effects on the bacteria. (a) Pyocyanin (PYO), (b) 1-Hydroxyphenazine (1OH-PHZ), (c) Phenazine-1-carboxylic acid (PCA) and (d) Phenazine-1-carboxamide (PCN).

6.2. Experimental Methods

To investigate the chemotactic effect of Phenazines on PA14WT, we first prepared the Phenazine solutions at concentrations 5, 10, 20 and 40 µg/ml. Then, the bacterium is prepared like we did for the motility in carbon sources experiment. PA14WT is inoculated from the LB agar plate into a test tube with 5ml LB stock solution and left overnight to grow in an incubator. 12-16 hours later, 200µl of the bacteria stock is transferred onto 5ml of 30mM 2,3-butanediol. Only 2,3-butanediol is used for the grow media since that is the carbon source of interest that may contribute to higher biofilm formation in BES. The tube is left overnight for about 16-24 hours. The liquid culture is sampled in a 200µl 96 well plate in triplicate to figure out their OD and then diluted or suspended, depending on the original OD, to 0.04 OD in 1ml tubes. This time, however, the culture is diluted (or suspended) in AB media only. This is to prevent multiple chemical gradient formations across the microfluidic device since the other chemicals will not be containing carbon sources. The bacteria are left aside to settle for about five to ten minutes.

Meanwhile, the hydrogel-based microfluidic device, which had been constructed the day before is prepared for experiments. The channels are flushed with the AB media. The Phenazine solutions are prepared in syringes with their outlets connected to plastic tubings connected to pipette tips that have been cut into size to fit the tubings. Another syringe with plastic tubing is prepared for AB media solution that will be the chemical in the sink channel. After removing air bubbles from the syringes, the syringes are loaded onto syringe pumps set at 5µl/min and the tips wedged into the inlets of the microfluidic device. Another pair of plastic tubing with the pipette tips cut to size connected to the end is wedged into the outlets of the device with the other tubing opening placed in a beaker for waste liquid collection. The setup is shown in Figure 9.

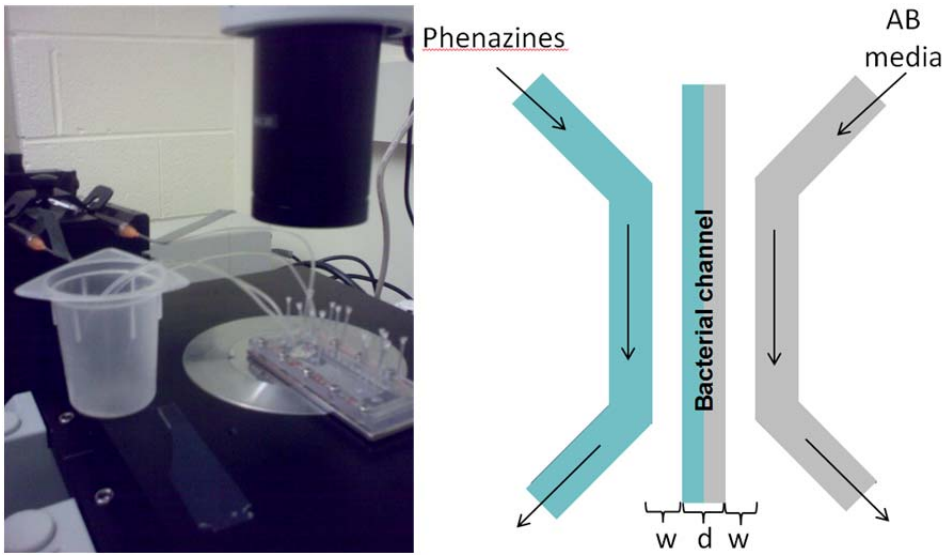


Figure 9: Phenazine chemotaxis experimental setup.
(a) Experimental setup with syringes in syringe pumps, microfluidic device connected to plastic tubings and a microscope overhead for imaging, (b) Schematic of the Phenazine chemotaxis experimental setup.

After the bacteria are loaded into one of the center channel of the device, the syringe pump is switched on. The diffusion coefficient of Phenazines is not known but we know that it has a molecular weight of 198 Da. This is comparable with glucose (molecular weight of 180 Da) with a known diffusion coefficient of 600$\mu m^2/s$. Using this data, we could estimate the chemical gradient establishing time as about 11.25 minutes. After the syringe pump is switched on, microscope imaging will begin at about 12-15 minutes. The imaging is taken at both 20x and 40x magnification. The 20x magnification offers a bigger picture of the center channel as we could observe if there is obvious chemotaxis in the channel. The 40x magnification, on the other hand, gives us a better imaging of PA14WT in the channel because they are not fluorescent like E. Coli. A 500-frame movie with a frame rate of 1/30 s is recorded. The bacteria-loading and microscope imaging procedure is repeated with the rest of the bacteria at different types and concentrations of Phenazines. It is important to do the bacteria-loading and microscope imagine step one at a time for each Phenazine of a particular concentration so that the bacteria will not slow down waiting too long in the channels.

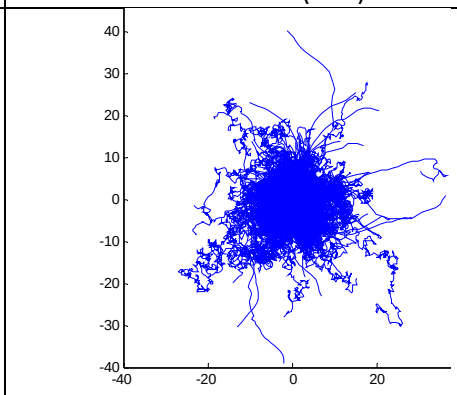
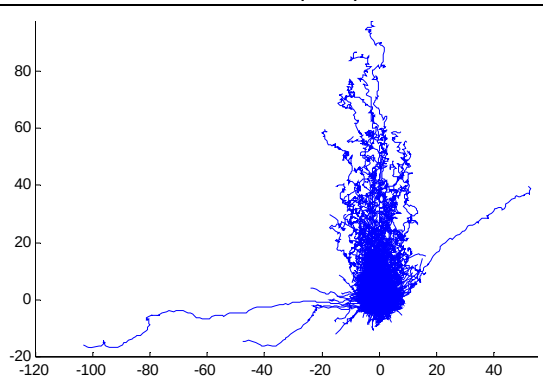
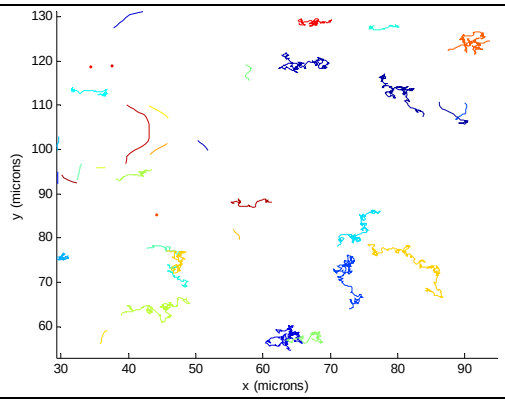
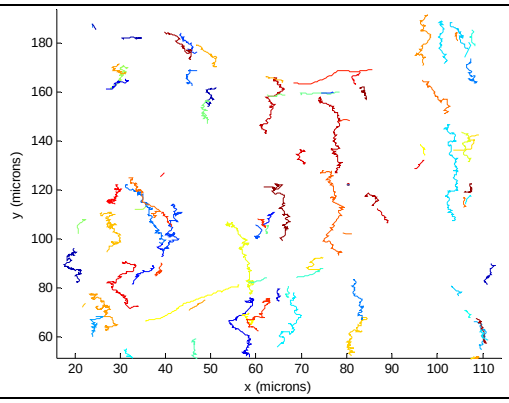
6.3. Results and Analysis

To analyze the movies, we used the 2D Tracking Program to create the tracks of the bacteria. Next, we plot the tracks in polar form to analyze the movement of any bacteria from a single common origin. In non-chemotactic environment, the polar plot will display evenly distributed bacterial tracks around an origin. In a chemotactic environment, however, the polar plot should show a skew in the positive y-direction (source channel) to indicate chemotactic effects of the chemical in the source channel on the bacteria.

Table 2 compares the polar plots and trajectories of a control experiment where there is no chemoattractant with those of 5µg/ml PCA Phenazine as the chemical in the source channel. The polar plot of 5µg/ml PCA Phenazine shows that it may be a potential chemoattractant to PA14WT due to its upwards skew indicating the effect of a chemoattractant on the swimming bacteria. The

bacterial trajectories also confirm this as the control experiment shows random swimming motion of the bacteria while the 5µg/ml PCA Phenazine experiment shows a general swimming motility of bacteria towards a single direction.

Table 2: PCA Phenazine is a potential chemoattractant to P. Aeruginosa

	Control: AB media (source) and AB media (sink)	5µg/ml PCA Phenazine (source) and AB media (sink)
Polar Plots		
Bacteria Trajectories		

Due to time limitation of this project, however, we could not be absolutely conclusive on the claim that Phenazines are chemoattractant to P. Aeruginosa. However, PCA Phenazine shows a very promising result (duplicated) that indicates the potential of Phenazines as a chemoattractant to PA14WT.

Smovie1 PA14 motility

7. CONCLUSIONS & FUTURE WORK

Some conclusions that can be derived from these sets of experiments include the following: (1) Carbon source enhances motility of P. Aeruginosa with glucose being a more potent carbon source and (2) PCA Phenazine shows promise to be a chemoattractant to P. Aeruginosa. Since more biofilm forms in the 2,3-butanediol MFC setup, and that P. Aeruginosa has a slower average velocity than glucose in general, it is possible that biofilm formation may be aided with slower-moving bacteria. In addition, maximizing current densities production in MFCs may be possible by having more slower-moving bacteria to form a thicker biofilm which translates to more Phenazine production for more efficient electron transfers. As of now, more work needs to be done to determine the chemotactic effect of Phenazines on the bacteria.

Other future work includes investigating the electrotaxis behavior of P. Aeruginosa. A simple experimental setup is shown in Figure 10. This would help answer questions regarding electron transfer mechanisms in the MFC systems.

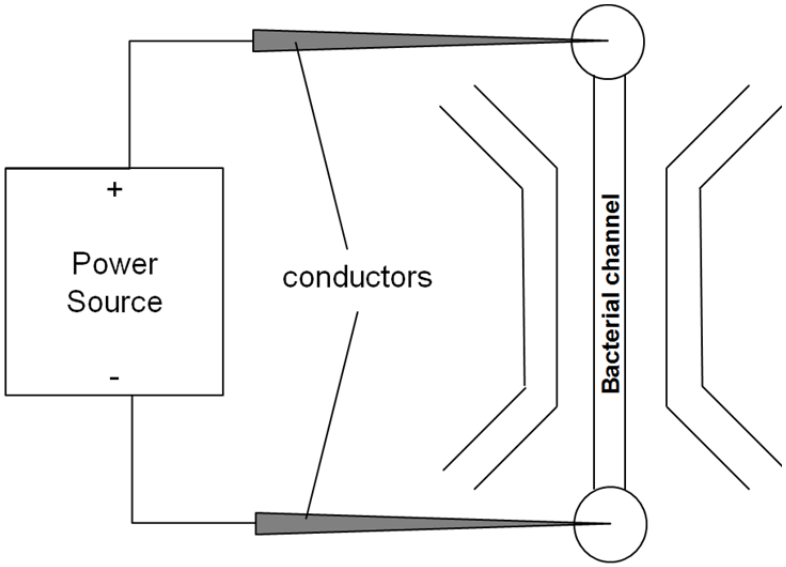


Figure 10: Future work – Electrotaxis experimental setup of P. Aeruginosa

8. ACKNOWLEDGMENTS

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