

EMERGING STRATEGIES IN BIOFILM CONTROL

By

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EMERGING STRATEGIES IN BIOFILM CONTROL

Abstract

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The objective of this dissertation was to develop new strategies in biofilm control. The first part of this dissertation covers the biofilm formation and biofilm-surface related problems in industrial systems. The second part of this dissertation covers the development of a new electrochemical flow cell to study bacteria surface interactions electrochemically. By using this flow cell system cell respiration can be determined. The third part of this dissertation focuses on the electrochemical biofilm control allowing dissection of the interactions between biofilms and stainless steel surfaces. Electrochemical method shows that the biofilm formation on these surfaces can be prevented. The fourth part of this dissertation is about biofilm cell density and effectiveness of the antimicrobial treatments in biofilm control. Finally the last part focuses on the future work for the cell respiration and antimicrobial control. Concluding remarks are also added to summarize the goal and focus of this dissertation.

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CHAPTER 1

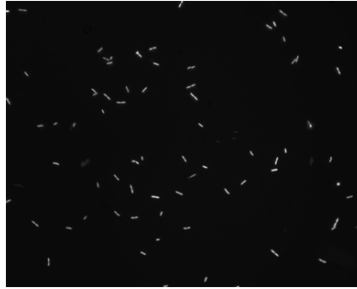
INTRODUCTION

1. Background

Biofilms are consortia of microorganisms in an extracellular polymeric substance (EPS) that survive and continue their life cycles. Attached cells persist in harsh environmental conditions and survive. When microorganisms form biofilms, they can express different genes, produce essential proteins to adapt to harsh conditions and ensure survival (Gandhi and Chikindas 2007; Fratamico, Annous B.A. et al., 2009). This unique ability of microorganisms can be employed in wastewater treatment (Vanloosdrecht and Heijnen, 1993), bioremediation (Singh, Paul et al., 2006) or in microbial fuel cells (Logan, Hamelers et al., 2006). However, they can also pose dangers in public health by contributing to food poisoning, the formation of dental (Socransky and Haffajee, 2002), or catheter biofilms (Donlan, 2001), or produce environmental hazards such as microbially induced corrosion (Beech and Sunner, 2004).

Biofilm formation is a process that starts with an initial attachment. Microorganisms require a substratum to attach and form mature biofilms. As seen in Figure 1, after the initial attachment, countable cells stay on the surface and remain attached irreversibly to glass cover slip. Finally, the cells grow on the surface and become mature biofilm.

t=0h



t=18h



t=36h

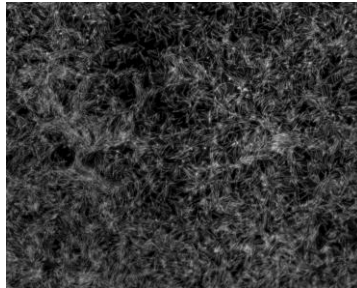


Figure 1. Biofilm formation steps of *P. aeruginosa* PAO1 on glass cover slip.

Biofilm surface interactions occur in various industrial systems. For instance, biofilms in the food industry cause huge losses in efficiency, lead to down time of processes, damage equipment, obstruct pipelines and decrease the competence of heat exchangers, create post-processing contamination that lowers shelf life and transmits diseases that eventually cost millions of dollars annually. Biofilm formation in food safety needs to be investigated more thoroughly to develop control methods in food processing systems.

Biofilm formation depends on the type of bacterial cells, attachment surfaces and the surrounding environment (Lewandowski and Beyenal, 2007; Van Houdt and Michiels, 2010). Given these components, food processing systems are very convenient environments for biofilm formation. Microorganisms can enter the food chain at different stages, adapt to the environment and, by forming biofilms, they may pose food safety risks (Mossel, Weenk et al. 1998; Havelaar, Brul et al. 2010; McMeekin, Hill et al. 2010). Besides health concerns, biofilm formation in food processing systems can cause metal corrosion in pipelines and tanks, reduce the heat transfer efficiency in heat exchangers, and cause mechanical blockages (Mittelman 1998). Many food industry sectors have their own microbial flora, depending on the food material that is processed, and the biofilms that form are specific to each processing system. For example, *Campylobacter*, *Salmonella*, *L. monocytogenes* and *E. coli* O157:H7 are found in red meat, poultry and seafood processing facilities; *Pseudomonas*, *Streptococcus*, *Bacillus* and *L. monocytogenes* in dairy processing plants; *Acinetobacter*, *Lactobacillus*, *Pediococcus* and *Dekkara* in brewing systems; *C. botulinum*, *L. monocytogenes*, *Salmonella*, *Shigella*, *E. coli* and *B. cereus* in fresh fruit and vegetable processes (Fratamico, Annous B.A. et al. 2009). *L. monocytogenes* is a gram-positive, motile, facultative anaerobic, food-borne pathogen (Todhanakasem and Young 2008) that is a particular concern in various food industries for its ability to form biofilms, detach from the biofilm and contaminate the food products (Borucki, Peppin et al. 2003; Marsh, Luo et al. 2003; van der Veen and Abee 2010). For example, in dairy processing systems, raw milk can be contaminated, causing the contamination of other dairy processing equipment and products such as cheese (Gay and Amgar 2005; Perni, Aldsworth et al. 2007; Guillier, Stahl et al. 2008; Alessandria, Rantsiou et al. 2010; Latorre, Van Kessel et al. 2010). Contamination may also occur via slicer blades in delicatessen meats such as turkey and salami (Keskinen, Todd et al.

2008; Keskinen, Todd et al. 2008). Moreover, depending on other microorganisms found in the environment, such contamination can actually be protected from environmental stresses. It was reported by Rieu *et al.* (Rieu, Lemaitre et al. 2008) that *L. monocytogenes* EGDe sessile populations were higher in dual species biofilms formed with the CIP 53.156 strain of *S. aureus*. Currently, the Food and Drug Administration has a zero-tolerance policy for *L. monocytogenes* in ready-to-eat foods, and the detection of any *L. monocytogenes* in a food makes the product adulterated (Gandhi and Chikindas 2007).

Antimicrobial agents commonly used in biofilm control are chemical or biochemical compounds that inhibit or kill microorganisms. Antimicrobials called antibiotics are used against infections in humans and animals (Fratamico, Annous B.A. et al., 2009). Biocides are used in disinfecting inanimate objects such as food processing surfaces. The chemical methods used in the food industry to control biofilms (Kumar and Anand, 1998) involve chemical agents, capable of destroying living organisms: for example, disinfectants such as hydrogen peroxide, quaternary ammonium compounds, and chlorine. (Pan, Breidt et al., 2006; Van Houdt and Michiels, 2010). Biocides are effective on planktonic cells; however, when biofilms form, biocides may not be as effective in destroying bacteria (Shi and Zhu, 2009; Simoes, Simoes et al., 2010; Van Houdt and Michiels, 2010), possibly due to the EPS matrix (Van Houdt and Michiels, 2010) including diffusion barriers, differential metabolic activity, and cell-wall ultra structures (Mittelman, 1998). It was shown by Stewart *et al.* (Stewart, Rayner et al., 2001) that *P. aeruginosa* PAO1 and *K. pneumonia* KP1 biofilm microorganisms had higher resistance to disinfectants when compared to their planktonic cells. In their study, alkaline hypochlorite or chlorosulfamate at a concentration of 1000 mg/L was applied both to biofilm and to planktonic bacteria; the

planktonic bacteria were easily killed, but biofilm bacteria were only partially killed. However, they also suggested that poor antimicrobial penetration was an insufficient explanation for biofilm resistance, because, although they found that the antimicrobial agent they employed effectively penetrated the biofilm, the results for biofilm killing were still poor. This suggests that bacteria in the biofilm were protected by some mechanism other than simple physical shielding by the biofilm matrix. Frank *et al.* (Frank and Koffi, 1990) stated that, after the combined treatment of sodium hypochlorite and heat, mean reduction values in viable counts were about 100 times lower for *L. monocytogenes* biofilms than for planktonic cells. In another study (Folsom and Frank, 2006), thirteen strains of *L. monocytogenes* biofilms grown on stainless steel coupons were treated with 60 mg/L chlorine solution. Eleven of these biofilms survived after being exposed to the chlorine solution. Robbins *et al.* (Robbins, Fisher et al., 2005) studied the efficacy of ozone, chlorine and hydrogen peroxide on *L. monocytogenes* Scott A strain in both planktonic and biofilm form. They found that a 16-fold increase in sanitizer concentration was required to destroy biofilm cells compared to planktonic cells. Depending on the type of biocide used, the effectiveness of the biocide to remove biofilms can vary. In one study (Vaid, Linton et al., 2010), four day-old biofilms were formed from a mixture of five *L. monocytogenes* strains (Scott A, N1-227, 103M, 82, and 311) on stainless steel coupons. The effect of chlorine dioxide (CD) gas, aqueous CD and aqueous sodium hypochlorite on these biofilms were investigated. It was found that 0.3 mg/L CD gas, 7 mg/L aqueous CD and 50 mg/L sodium hypochlorite treatment of 10 min resulted in 3.21, 3.74, 3.09 log₁₀ CFU/cm² reductions, respectively, from 4.80, 5.09, 4.95 log₁₀ CFU/cm² of initial populations of biofilms respectively. Moreover, when a multi-culture biofilm is developed, the resistance to biocide can be increased. Norwood *et al.* (Norwood and Gilmour, 2000) investigated the effect of sodium hypochlorite on

28 day old biofilms of *L. monocytogenes* when grown in a multi-culture with *P. fragi* and *S. xylosus*. They obtained a 2-log reduction in bacterial numbers after a 1000 mg/L free chlorine treatment, whereas 10 mg/L of the same treatment was able to eliminate all the bacteria in the culture.

Furthermore, the cleaning and disinfecting efficiency on bacteria also depends on the food matrix, since the soiling of the surface provides a convenient environment for bacteria to easily attach to and develop biofilms on. A study by Gram *et al.* (Gram, Bagge-Ravn et al., 2007) showed that when *L. monocytogenes* biofilm grown on stainless steel coupons in a fish broth system was treated with cleaning and disinfectant agents (MC 103 or FC140, and Oxivit Active Plus), all bacteria were killed. A 2-3 log of bacteria were killed by alkaline cleaning products (MC 103 or FC140) when a peracetic acid based disinfectant (Oxivit Active Plus) was used, and all bacteria were killed in salmon emulsions, whereas a 1-2 log were killed in meat emulsions. In another study (Olmez and Temur, 2010), *E. coli* and *L. monocytogenes* biofilms on lettuce leaves were treated at 10 °C for 2 min with ozone (2 mg/L), chlorine (100 mg/L) and organic acid (0.25 g/100g citric acid plus 0.50 g/100g ascorbic acid). They found that none of the treatments were effective in removing biofilms.

The interactions between bacteria and solid surfaces, however, are very complex and challenging. In order to study the mechanisms of these interactions, well-defined systems should be used. The objective of my dissertation was to develop novel methods to control biofilms in various biofilm systems. The first part of my project concerns bacterial cell respiration on solid surfaces where there is no electron acceptor except the electrode surface. The second part focuses on an electrochemical method in which we studied the mechanism of preventing biofilm

formation on stainless steel surfaces. The last part deals with the mechanism of antibiotic resistance in biofilm control. In the rest of this chapter, the motivations and expectations of my work are discussed.

2. Monitoring metabolic rates of individual electrode-respiring cells using a real-time imaging electrochemical flow cell

It is well known that certain bacteria can respire anaerobically on solid conducting materials, using extracellular electron transfer (EET) (Gralnick and Newman 2007; Logan 2009; Borole, Reguera et al. 2011). This ability is important for subsurface biogeochemical processes, microbiologically influenced corrosion, and microbial fuel cell research. For example, cells respiring on electrodes are used in microbial fuel cells to generate electrical current (Potter 1911; Logan and Regan, 2006; Lovley, 2006; Lovley, 2008); cells in sediments can dissolve metals and play a critical role in subsurface biogeochemistry (Coleman, Hedrick et al., 1993; Nevin and Lovley, 2002; Lovley, Holmes et al., 2004); and cells growing on metal surfaces can influence corrosion (Coetser and Cloete 2005; Herrera and Videla 2009). Cells capable of EET start respiring on the relevant surfaces as soon as they attach to the surface. Most of the time, attached cells mature and develop into biofilms; therefore, the respiration of biofilms on electrode surfaces has generally been the chosen method to investigate EET. However, cellular respiration and electrode-cell interactions at the initial step of cell attachment have primarily been ignored due to very small electron transfer rates and difficulties associated with *in-situ* imaging of cells on solid electrodes.

There are several works focusing on the development of *in-situ* monitoring systems. Mc Lean et al. (2010) have developed a method to quantify electron transfer rates to an electrode through the stages of biofilm formation, from single cells up to multicellular communities (McLean, Wanger et al., 2010). In this work they used *Shewanella oneidensis* strain MR-1 p519nGFP (constitutively expressed green fluorescent protein), a model species capable of respiration on solid surfaces serving as an electron acceptor. A significant drawback of this system was that it was operated as a microbial fuel cell where the anode and cathode were connected through a resistor, allowing for the studied electrode (anode) potential to change over time. A new method which uses the electrode as the sole electron acceptor (without any other soluble electron acceptor) allowing *in-situ* imaging is required. A better system should be able to be connected to a potentiostat to control the working electrode potential, quantifying electron transfer rates as current, and allowing for real-time imaging of individual cells. A system capable of *in-situ* monitoring also would allow me to control surface potential and study cell growth as discussed in the following chapters.

The goals of chapter 2 were 1) to quantify *in-situ* single cell electron transfer rates on an electrode surface when the electrode was the electron acceptor, and 2) to quantify open circuit potential of the electrode and correlate it with the cell growth. We have developed a well-controlled electrochemical flow cell that allowed us to image the cells on the surface, quantify potential of the electrode, and polarize another electrode to quantify electron transfer all in real-time. The electrochemical cell we used was analogous to a microbial fuel cell, but the potential of the working electrode was controlled by a potentiostat. We used an inverted microscope to monitor *in-situ* cell growth on the surface and count the cells. We measured the potential of a

non-polarized electrode against a Ag/AgCl reference electrode located in the cathodic compartment of the electrochemical cell.

Using our bioelectrochemical flow cell imaging system, we successfully and repeatedly grew *S. oneidensis* biofilms, monitored their growth and metabolic activity. For our purpose we define the measured current per cell as the cellular metabolic respiration rate since current shows the number of electrons transferred per unit cell, which is equivalent to their respiration rates on the electrodes. Experiments were reproducible in that every run we obtained statistically similar number of cells attached to each electrode and statistical tests demonstrated that the results were statistically similar. The results of this work detailed in Chapter 2.

Figure 2 shows cell respiration rates calculated as current per cell for the first day of cell attachment for different inoculation densities. We found that an inoculation optical density of 0.036 gave the maximum cell respiration rates, whereas higher inoculation densities decreased cell respiration rates. However, these rates leveled off (~ 8 fA/cell) for higher inoculation densities. Our results indicated that for the estimation of cell respiration rates, there must be a critical number of cells attached to the surface and for our system this number was $\sim 4 \times 10^6$ cells/cm².

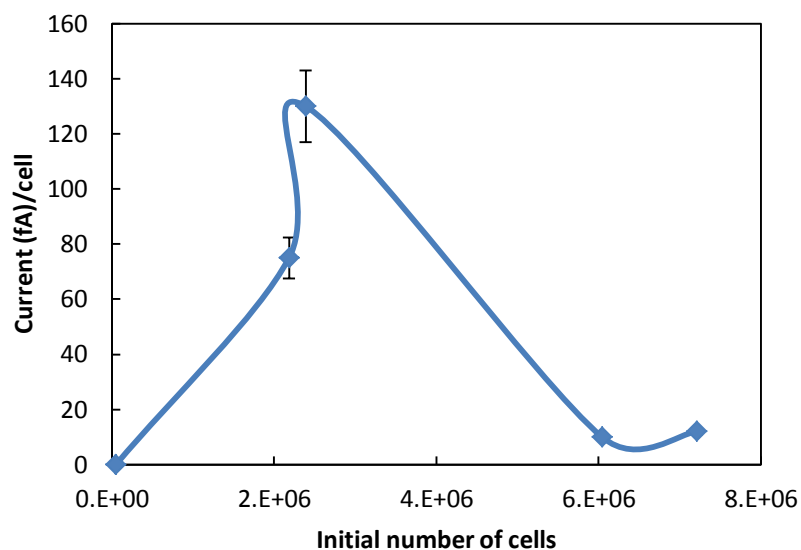


Figure 2. Cell respiration rate of *S. oneidensis* MR1 on glassy carbon for 24 h.

Our results demonstrated that the cells can grow on electrodes that are not polarized, even in anaerobic conditions. After the cells are attached to the surface, the open circuit potential of the electrode decreases as the populations grow. This decrease happens because of the electrons generated as a result of cell respiration which are deposited on the electrode surface acting as a capacitor, or more likely due to the change in concentration of interactable redox couples near the electrode surface. We concluded that 1) growth of the cells on the polarized and non-polarized electrodes were statistically similar, 2) cell respiration was inversely correlated to inoculum density. The denser the cell inoculation, the lesser is the cell respiration, 3) the potential of the non-polarized electrode was dependent on the inoculation density, and 4) maximum cell respiration rate (130 fA/cell) was observed when inoculation density was 2.39×10^6 cells/cm².

3. Electrochemical biofilm control

Chapter 3 presents the work of electrochemical biofilm control. In this study we focused on the mechanism of the bioelectrochemical biofilm control. Although electrical current has been previously demonstrated to control biofilm growth on metal surfaces, there is no single accepted mechanism of action (Del Pozo, Rouse et al. 2008). Some of the mechanisms proposed in the literature are 1) electrochemical generation of oxidants on the surface (Costerton, Ellis et al., 1994), 2) contraction or expansion of biofilm structure (Stoodley, deBeer et al. 1997), 3) electrolytic generation of oxygen during water electrolysis (Stewart, Wattanakaroon et al., 1999), and 4) electrostatic and electrophoretic repulsive forces (Hong, Jeong et al. 2008). However, none of these mechanisms is widely accepted because of conflicting results in the literature and the impossibility of comparing the different electrochemical systems. It is important to note that in the literature, different electrochemical cells with different geometries are operated using either a controlled potential—potentiostatic system—or a controlled current—galvanostatic system—to achieve the bioelectrochemical effect. Table 2 summarizes the literature about the systems used to evaluate the bioelectrochemical effect. We can see that all the systems differ from each other. Shirtliff et al. (2005) used an annular reactor with a stainless steel surface to evaluate the bioelectrochemical effect. They applied a $370\text{-}\mu\text{A}/\text{cm}^2$ current and found that the applied current did not have any bactericidal effect (Shirtliff, Bargmeyer et al. 2005). Similarly, Hong et al. (2008) used a flow chamber and applied a $15\text{-}\mu\text{A}/\text{cm}^2$ current and found that the applied current caused detachment of the biofilm on the indium tin oxide surface (Hong, Jeong et al., 2008). They hypothesized that this was due to electrostatic and electrophoretic repulsive forces. Similar observations were made by Van der Borden et al., (2004), who applied a current between 2.86 and $4.76\text{ }\mu\text{A}/\text{cm}^2$ (van der Borden, van der Werf et

al., 2004). Liu et al. (1997) inserted two electrodes into agar in a plate and found a zone where biofilm growth was inhibited (Liu, Brown et al., 1997) due to hydrogen peroxide production. On the other hand, Del Pozo et al. (2009) applied a current between 1.626×10^3 and 1.626×10^6 $\mu\text{A}/\text{cm}^2$ and did not detect hydrogen peroxide; instead they concluded that pH was the cause of decreased biofilm growth (del Pozo, Rouse et al., 2009).

However, regardless of the type of system, a specified current is required to understand the rate of reaction and a specified potential is required to assess the thermodynamic state of the system. To have reproducible or comparable data, both the potential (galvanostatic) and current (potentiostatic) data must be presented. Additionally, the potential of the electrode must be defined against a reference electrode. Therefore it is critical to use a well-defined electrochemical system to understand the mechanisms of action of the bioelectrochemical effect.

Table 1. Selected potentiostatic and galvanostatic studies of bioelectrochemical biofilm control of various microorganisms.

Bacteria	Current density/ Potential	Material type/ Surface area	Experimental system	Log reduction	Imaging/ Bacterial enumeration	Exposure time	Mechanism	Reference
<i>P. aeruginosa</i> PAO1	15 $\mu\text{A}/\text{cm}^2$	WE: ITO 6.5 cm^2 CE: ITO No RE	Flow cell reactor No membrane Constant current applied	Cell adhesion prevented ~81%	Live dead staining , plate counting and biofilm monitor by microscope	Polarization then initial attachment for 90 min	Electrostatic repulsion, H_2O_2 produced but not enough to kill the cells	Shim et al., 2011
<i>P. fluorescens</i> ATCC 17552	-0.5 V -0.2 V	WE: Gold covered (10-20 nm) glass coverslip CE: Platinum wire RE: Ag/AgCl (sat.KCl)	Thin film flow cell No membrane Constant polarization potential	Cell adhesion prevented ~90 % at -0.5 V ~80 % at -0.2 V	Microscopy, gray scale images	Polarization then initial attachment for 15 min	Electrostatic repulsive forces	Busalmen and Sanchez, 2001
<i>P. fluorescens</i> ATCC 17552	-0.5, -0.2, 0.1, 0.5, and 0.8 V	WE: Gold covered (10-20 nm) glass coverslip CE: Platinum wire RE: Ag/AgCl (3M NaCl)	Thin film flow cell No membrane Constant polarization potential	Cell growth at -0.2 V No cells adhered; clusters at -0.5 V	Phase contrast microscopy	2 min initial attachment then polarization for 3 or 8h	Electrostatic repulsion plus Oxygen reduction at -0.5 V therefore pH increase	Busalmen and Sanchez, 2005
<i>P. aeruginosa</i> PAO1	15 $\mu\text{A}/\text{cm}^2$	WE: ITO 6.5 cm^2 CE: ITO No RE	Flow cell reactor No membrane Constant current applied	~80% detachment	Live dead staining and biofilm monitor by microscope	90 min of initial attachment then polarization for 40 min	Electrostatic and electrophoretic repulsive forces	Hong et al., 2008
<i>S. epidermidis</i> HBH276	60 μA 100 μA	WE: 316 LVM SS 21 cm^2 CE: ITO No RE	Parallel plate flow chamber No membrane Constant current applied	37 % detachment 78 % detachment	Live dead staining and CSLM, biofilm monitor by microscope	200 min of initial attachment then polarization for 360 min	Electro-osmotic fluid flow directed to and from the surface	Borden et al., 2004
<i>V. alginolyticus</i>	0 V -0.2 V -0.6 V	WE: Conductive paint and TIN film electrode CE: Iron bar RE: Ag/AgCl	Nylon yarn fish nets of 50 cm length, coated with 50 μm conductive paint	0 desorption ~14 % desorption ~48 % desorption	Live dead staining fluorescent microscopy	30 min	No toxic substance generation, electrochemical sterilization	Matsunaga and Lim, 2000
<i>S. aureus</i> Xen 30 <i>S. epidermidis</i> Xen 43 <i>P. aeruginosa</i> Xen 5	20 mA 200 mA 2000 mA	E: Stainless steel or Graphite cylinders (D: 1.5mm, L: 55 mm)	Biofilms grown on Teflon disks for 48h then placed to biofilm treatment device Constant current applied	4 to 5 log CFU/ cm^2 6 log CFU/ cm^2 3.5 to 5 log CFU/ cm^2	Plate counting	At least 2 days At least 2 days 7 days	No H_2O_2 detected. pH changes may be involved	Del Pozo et al., 2009
<i>S. epidermidis</i> <i>S. aureus</i>	10 μA 100 μA	Anode: Catheter Cathode: Catheter	Anode and cathode inserted into an agar plate inoculated with bacteria		Zone of inhibition test	16 h	A salt-bridge used; H_2O_2 was produced at the cathode	Liu et al., 1997
<i>P. aeruginosa</i> EX226	9 mA/ cm^2		Electrical colonization cell (ECC), biofilm formed onto dialysis membrane		Fluorescent microscopy, SEM	1-2 h initial colonization then 12h treatment		Jass and Lappin-Scott, 1996
Mixed-species water biofilm	3.7 $\mu\text{A}/\text{mm}^2$	Anode: Stainless steel Cathode: Stainless steel	Annular reactor	No bactericidal action	Plate counting	7 days	No bactericidal action	Shirliff et al., 2005

In electrochemical systems, the main controlled parameter is potential. By knowing the potential, we can predict electrochemical reactions occurring on the surface. To investigate the ability for electrochemical systems to prevent biofilm formation, it is critical to use a potential controlled system. A potentiostat can be used to control the potential of a metal electrode, which will be the surface that biofilms will be grown on, against a standard reference electrode and measure current. Our motivation was to develop a well-defined system for biofilm control. Therefore, initially we developed the bioelectrochemical system and then tested its efficiency. We considered that if oxygen reduction takes place cathodically we should be able to measure the product at the surface when we used polarization at $-600 \text{ mV}_{\text{Ag/AgCl}}$.

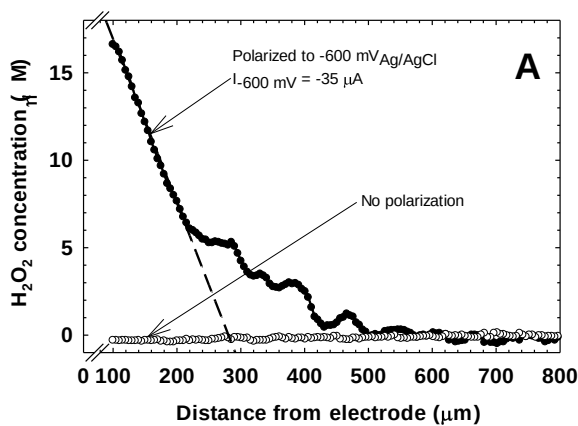


Figure 3. Hydrogen peroxide profiles above both non-polarized SS surfaces (○) and polarized SS surfaces (●).

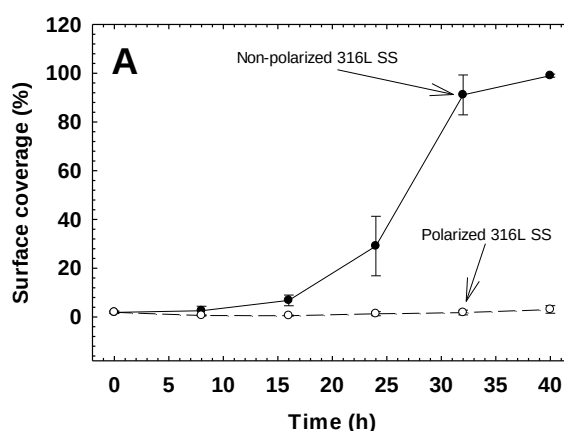


Figure 4. The variation in surface coverage of polarized (○) and non-polarized (●) 316L SS surfaces over time by *P. aeruginosa* PAO1.

Our goals were 1) to develop a well-controlled system to explain the mechanism of action of the bioelectrochemical effect on 316L stainless steel (SS) surfaces, 2) to develop a technology to measure the hydrogen peroxide concentration 100 μm above polarized SS surfaces, and 3) to test whether the produced hydrogen peroxide can reduce cell growth on metal surfaces. One of the suggested mechanisms in the literature is the production of hydrogen peroxide on metal surfaces; surprisingly these studies showed that hydrogen peroxide could not be detected in the bulk solution (del Pozo, Rouse et al., 2009). We found that hydrogen peroxide was produced near 316L SS surfaces when a negative potential was applied (Figure 3). The hydrogen peroxide concentration increased towards the surface, while the dissolved oxygen decreased when the SS surface was polarized to $-600 \text{ mV}_{\text{Ag/AgCl}}$. When polarized and non-polarized surfaces with identical *Pseudomonas aeruginosa* PAO1 biofilms were continuously fed, the polarized surfaces

showed insignificant biofilm growth while there was significant biofilm growth on the non-polarized surfaces (Figure 4). Findings for this method are presented in details in the Chapter 3.

4. Antibiotic mass to Colony Forming Unit (CFU) ratio determines effectiveness of an antibiotic against biofilms and planktonic cells

Chapter 4 presents a new aspect of health related biofilm control with antibiotics. Several mechanisms are proposed in the literature in terms of resistance of biofilms to antibiotics. Antibiotic resistance in biofilms can be attributed to the following factors; 1) slow or incomplete penetration of antibiotics into the biofilm (Harmsen, Yang et al., 2010), 2) development of a resistant phenotype (Stewart and Costerton, 2001), 3) adaptive responses (Stewart, 2002), 4) quorum sensing signaling (Simones, 2010), 5) genetic transfer, expression of resistance genes (Stewart, 2002), and 6) persisters (Lewis 2007). However, none of these mechanisms are sufficient to explain the fact that we always need to use at least 1000 times more antibiotics for cells in biofilm compared to planktonic cells. The fact that the fact that biofilm communities are more densely packed than planktonic cells is often overlooked. Focusing on this fact, we hypothesized that biofilms are more resistant against antibiotics compared to planktonic cells because the amount of antibiotics per unit cell weight is less for biofilms when biofilms were treated with the same concentration of antibiotics. Our goal was to demonstrate that the log reduction was dependant on the amount of antibiotic used *per number of cells*. For this purpose, we used *Pseudomonas aeruginosa* due to the severity and number of diseases it causes and the presence of extensive research on these bacteria. We selected tobramycin as an antibiotic since it is used against *P. aeruginosa*. The planktonic and biofilm samples were tested against different

tobramycin concentrations. We used chemostat for the planktonic cells and we designed hollow fiber biofilm reactors (HFBRs) to grow biofilms. By using HFBRs, we eliminated the retarded antibiotic diffusion into the biofilms as well as the oxygen limitation as potentially confounding variables. As expected, log reduction was a function of antibiotic amount per cell density (Figure 5) for the planktonic cells.

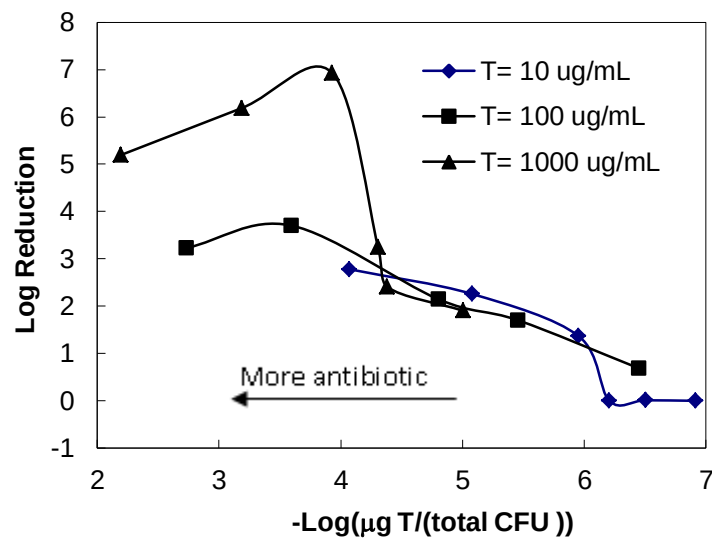


Figure 5. The $-\log(\mu\text{g tobramycin}/\text{CFU})$ versus log reduction for *P. aeruginosa* PAO1 cells from chemostat.

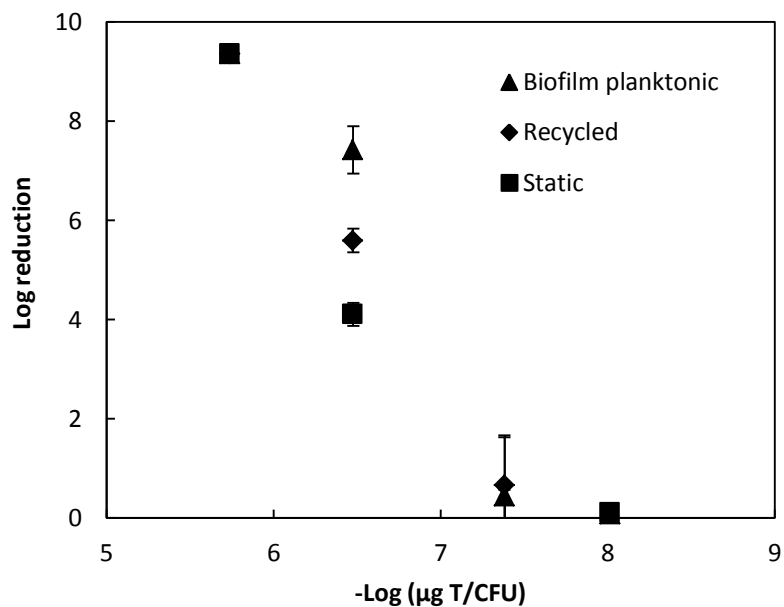


Figure 6. The $-\log (\mu\text{g tobramycin/CFU})$ versus log reduction for various tobramycin concentrations for different treatments.

When we challenged the biofilms with tobramycin we found similar results to that of the planktonic cells (Figure 6). When we treated the biofilms in recycled and non-recycled modes, we found a zone in which the log reduction was dependant on the amount of tobramycin used per CFU. However there was no zone for the higher and lower concentrations. The details of this study are given in Chapter 4.

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CHAPTER 2

MONITORING METABOLIC RATES OF INDIVIDUAL ELECTRODE-RESPIRING CELLS USING A REAL-TIME IMAGING BIOELECTROCHEMICAL FLOW CELL

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In this work, I developed the bioelectrochemical flow cell and conducted the experiments.

Abstract

Microorganisms can grow in the presence of an electron acceptor and an electron donor which are supplied in soluble forms. However, some microorganisms can use electrodes as solid electron acceptors. The respiration rates can be quantified by measuring electron transfer rate per cell or current per cell when single cells were growing on the electrode surface. During experiments, we noticed that some attached cells to the electrode surfaces can grow without passing a current on a non-polarized electrode. We hypothesized that the cells that attach to the electrodes can still grow by depositing electrons onto the electrode surface and changing the open circuit potential of the electrode. To test this hypothesis, we developed a bioelectrochemical system that monitors both the *in-situ* cell growth and potential of the electrodes against a reference electrode, and also quantifies the current of a polarized electrode. As a model microorganism, we used *Shewanella oneidensis* strain MR-1 using lactate as an electron donor and a glassy carbon electrode as the electron acceptor under anaerobic conditions. We found that cells on the polarized and non-polarized electrodes grew at a similar rate. The cell respiration rates depended inversely on the inoculum density, i.e., more initially attached cells caused less cell respiration. Calculated single cell respiration rate depended on the inoculation density and the maximum observed respiration rate was 130 fA/cell and the minimum was 8 fA/cell.

Keywords: Electron transfer, single cell, electrode, respiration, biofilm, *Shewanella oneidensis*

1. Introduction

It is well known that certain bacteria can respire anaerobically on solid conducting materials using extracellular electron transfer (EET) (Gralnick and Newman 2007; Logan 2009; Borole, Reguera et al. 2011). This ability is important for subsurface biogeochemical processes, microbiologically influenced corrosion, and microbial fuel cell research. For example, cells respiring on electrodes are used in microbial fuel cells to generate electrical current (Potter 1911; Logan and Regan 2006; Lovley 2006; Lovley 2008), cells in sediments can dissolve metals and play critical a role in subsurface biogeochemistry (Coleman, Hedrick et al. 1993; Nevin and Lovley 2002; Lovley, Holmes et al. 2004), and cells growing on metal surfaces can influence corrosion (Coetser and Cloete 2005; Herrera and Videla 2009). Cells capable of EET start respiring on the relevant surfaces as soon as they attach to the surface. Most of the time, attached cells mature and develop into biofilms and therefore respiration of biofilms on electrode surfaces has generally been the chosen method to investigate EET. However, cellular respiration and electrode-cell interactions at the initial step of cell attachment has primarily been ignored due to very small electron transfer rates and difficulties associated with *in-situ* imaging of cells on solid electrodes.

Recently, we have developed a method to quantify electron transfer rates to an electrode through the stages of biofilm formation, from single cells up to multicellular communities (McLean, Wanger et al. 2010). In this work, we used *Shewanella oneidensis* strain MR-1 p519nGFP (constitutively expressed green fluorescent protein), a model species capable of respiration on solid surfaces serving as an electron acceptor. A significant drawback of our system was that it operated as a microbial fuel cell where the anode and cathode were connected through a resistor,

allowing for the studied electrode (anode) potential to change over time. A new method which uses the electrode as the sole electron acceptor (without any other soluble electron acceptor) allowing *in-situ* imaging is required. A better system should be able to be connected to a potentiostat to control the working electrode potential, quantify electron transfer rates as current, and allow for real-time imaging of individual cells.

When an electrode is exposed to microorganisms, the electrode shows a different potential profile over time when compared to sterile growth medium (Xu, Duan et al. 2010; Renslow, Donovan et al. 2011). The cells attached to the surface change the potential of the electrode. For *S. oneidensis* MR-1, the depression of the electrode potential is dependent upon the mediators expressed to transfer electrons. These mediators, known to be bound to the cell surfaces, have a higher concentration as more cells colonize the electrode surface. In other words, cell numbers on the surface can be linked to the development of the electrode potential over time. In a simplistic approach, the electrode potential can be described as a pseudo-Nernst response where electrode potential is determined by the cell surface coverage (ϕ). Equation 1 introduces cell coverage into the electrode potential dependence by assuming that mediators are only present on the cell surfaces.

$$E_{OCP} = (1 - \phi)E_{initial} - (\phi)\frac{59}{n} \log \frac{[Med_{RED}]}{[Med_{OX}]} \quad \text{Eq 1}$$

This assumption can only be true for young biofilms with minimal EPS production since it is known that mediators are present in the biofilm matrix. This type of mechanistic approach has not been done before. However, steady state measurements of electrode potential after *S. oneidensis* biofilm growth revealed a limiting potential of $-470\text{mV}_{\text{SCE}}$. The typical electrode potential in sterile growth medium is approximately $+200\text{ mV}_{\text{SCE}}$ (Renslow, Donovan et al. 2011). What is missing is the critical cell surface coverage that is required to reach the limiting electrode potential. This information is crucial to understand how electron flux to a solid conductor first develops after initial attachment.

The goals of this study were 1) to quantify *in-situ* single cell electron transfer rates on an electrode surface when the electrode was the electron acceptor, and 2) to quantify open circuit potential of the electrode and correlate it with the cell growth. We developed a well-controlled bioelectrochemical flow cell that allowed us to image the cells on the surface, quantify the open-circuit potential of an electrode, and polarize another electrode to quantify electron transfer all in real-time. The bioelectrochemical flow cell we used was analogous to a microbial fuel cell, but the potential of the working electrode was controlled by a potentiostat. We used an inverted microscope to monitor *in-situ* cell growth on the surface and count the cells. We measured the potential of a non-polarized electrode against a Ag/AgCl reference electrode located in the cathodic compartment of the bioelectrochemical cell.

2. Materials and methods

2.1. Preparation of growth medium and inocula

Shewanella oneidensis MR-1 stock cultures, stored in 1 mL vials at -70 °C, were thawed at room temperature (approximately 25 °C) and used to inoculate sterilized 100 mL M1 minimal medium (described below). The culture was incubated on a shaker at 150 rpm at room temperature. After 24 hours, 1 mL of the culture was transferred into a quartz cuvette to measure the optical density at 600 nm (OD₆₀₀). The culture was then used to create a set of diluted inocula in 50 mL pre-sterilized test tubes with sterilized M1 minimal medium. The set consisted of inocula with optical densities fixed to 0.005, 0.013, 0.036, 0.301 and 0.402 at OD₆₀₀.

M1 minimal medium was prepared with deionized water in a carboy, and included pipes buffer (C₈H₁₈N₂O₆S₂) 0.91 g/L, sodium hydroxide (NaOH) 0.3 g/L, ammonium chloride (NH₄Cl) 1.5 g/L, potassium chloride (KCl) 0.1 g/L, sodium phosphate monobasic (NaH₂PO₄H₂O) 0.6 g/L, sodium chloride (NaCl) 1.75 g/L, and mineral solution 10 mL/L. The pH of the medium was set to 7.0 using HCl or NaOH as needed. A polytetrafluoroethylene membrane filter was spliced to one of the ports of the carboy lid to allow for aseptic venting. The medium was autoclaved at 121 °C for 20 minutes. After allowing the medium to cool, 10 mL/L vitamin solution, 10 mL/L amino acid solution, and 1.1206 g/L sodium lactate [60 % (w/w)] in distilled water were added aseptically in a biosafety hood.

Mineral Solution: Nitrilotriacetic acid 1.5 g/L, magnesium sulfate heptahydrate 3 g/L, magnesium sulfate monohydrate 0.5 g/L, ferrous sulfate heptahydrate 0.1 g/L, calcium chloride

dihydrate 0.1 g/L, cobalt chloride hexahydrate 0.1 g/L, zinc chloride 0.13 g/L, cupric sulfate pentahydrate 0.01 g/L, aluminum potassium disulfate dodecahydrate 0.01 g/L, boric acid 0.01 g/L, sodium molybdate dihydrate 0.025 g/L, nickel chloride 0.024 g/L, and sodium tungstate 0.025 g/L added to deionized water. The pH was set to 7.0 by using HCl or NaOH as needed. The solution was filter-sterilized and kept refrigerated.

Vitamin solution: Biotin 0.002 g/L, folic acid 0.002 g/L, pyridoxine HCl 0.01 g/L, riboflavin 0.005 g/L, thiamine HCl monohydrate 0.005 g/L, nicotinic acid 0.005 g/L, d-pantothenic acid hemicalcium salt 0.005 g/L, B12 0.0001 g/L, p-aminobenzoic acid 0.005 g/L, and thiocctic acid 0.005 g/L added to deionized water. The pH was set to 7.0 by using HCl or NaOH as needed. The solution was filter-sterilized and kept refrigerated.

Amino acid solution: L-glutonic acid 2 g/L, L-arginine 2 g/L, and DL-serine 2 g/L added to distilled water. The pH was set to 7.0 by using HCl or NaOH as needed. The solution was filter-sterilized and kept refrigerated.

2.2. Preparation and operation of bioelectrochemical flow cell

For this study, a custom-built bioelectrochemical flow cell (Figure) was designed to allow for simultaneous *in situ* monitoring of cell growth, quantification of metabolic rates per cell measured as current, and measuring of electrode open circuit potential (OCP). It consisted of two adjacent parallel flow chambers: An anodic compartment for the working (polarized) and OCP-measuring (non-polarized) electrodes, and a cathodic compartment for the reference and counter

electrodes. The flow cell was constructed out of polycarbonate and the chambers were separated by a cation exchange membrane (Membranes International, CMI-7000). Glassy carbon electrodes (1×1 cm), used for the working and OCP-measuring electrodes, were prepared by washing with deionized water and sonicating for 1 h in 1 M HCl solution, and then air drying. Platinum wires were connected to the electrodes by conductive epoxy, which was subsequently covered with silicon. A Ag/AgCl wire was used as the reference electrode and a graphite plate acted as the cathode (counter electrode). High purity nitrogen was fed into the M1 minimal medium prior to inoculation to ensure anaerobic conditions. The medium was pumped to the anodic compartment of the bioelectrochemical flow cell at 0.4 mL/min. In the cathodic compartment, sterile catholyte solution (3 M NaCl) was pumped at the same flow rate (0.4 mL/min). After the OCP of both anodic compartment electrodes reached steady values and the system was completely anaerobic, the feed was stopped for inoculation. For each individual experiment the flow cell was inoculated with 10 mL of one of the inocula (0.402, 0.301, 0.036, 0.013, or 0.005 at OD₆₀₀) using a pre-sterilized syringe. After 2 h of initial attachment, the medium was again pumped through the system. A Gamry Reference 600™ potentiostat was used to polarize the working electrode to +400 mV_{Ag/AgCl}. Current values were continuously monitored and recorded by computer. Cyclic voltammograms of the electrodes were obtained using a Gamry Reference 600™ potentiostat as needed at 10 mV/s from -900 to +500 mV_{Ag/AgCl}.

We were able to image cells attached to the polarized and non-polarized electrodes through the glass cover slip located at the bottom of the flow cell. Images were captured by a Nikon DS-Qi1Mc camera mounted on a brightfield-inverted microscope (Nikon Eclipse Ti-S) with a 40x extra-long working distance (ELWD) objective. At least 20 images were taken during each

imaging event. In our previous work (Ica, Caner et al. 2012), we found that taking 10 images generated statistically reproducible data. We manually counted the number of cells in each image and then calculated the number of cells per unit surface area.

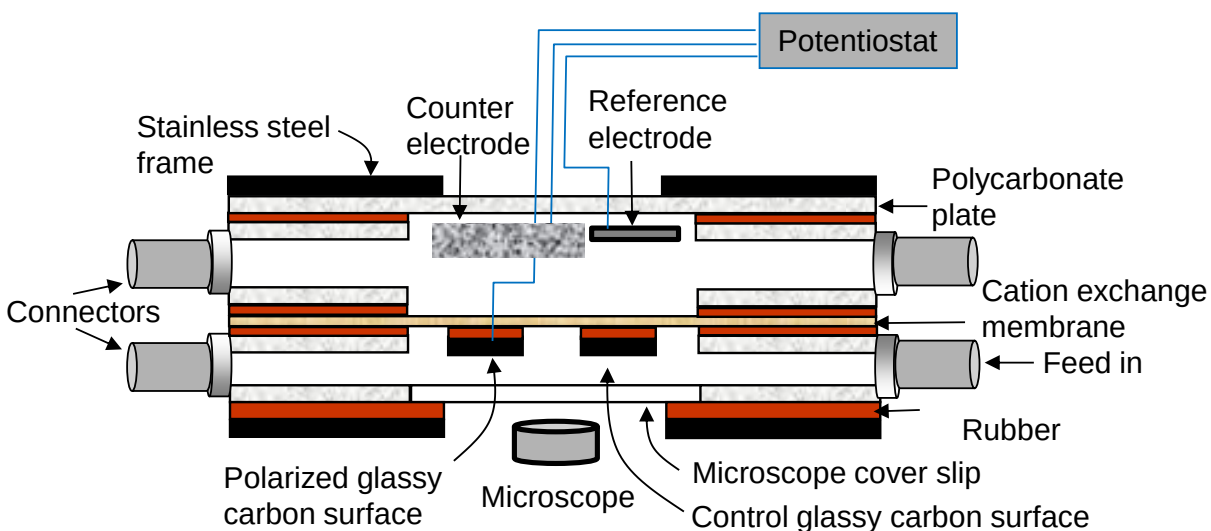


Figure 1. A schematic of the experimental configuration and bioelectrochemical flow cell.

The figure is not drawn to scale.

3. Results and discussion

3.1. Cell attachment and growth on the electrodes

Using our bioelectrochemical flow cell imaging system we successfully and repeatedly grew *S. oneidensis* biofilms, and monitored their growth and metabolic activity. In this paper, we defined the measured current per cell as the cellular metabolic respiration rate since current shows the number of electrons transferred per unit cell, which is equivalent to their respiration rates on the electrodes. Experiments were reproducible in that in every run we obtained statistically similar

numbers of cells attached to each electrode. One example data set is shown in Figure 1, demonstrating attached cells on the polarized and non-polarized electrodes. This Figure shows that the numbers of attached cells on the surface increases by time. The reported current values refer to average respiration rates of single cells on the surface. During the growth of the cells in Figure 1 the current was $\sim 0.3 \mu\text{A}/\text{cm}^2$.

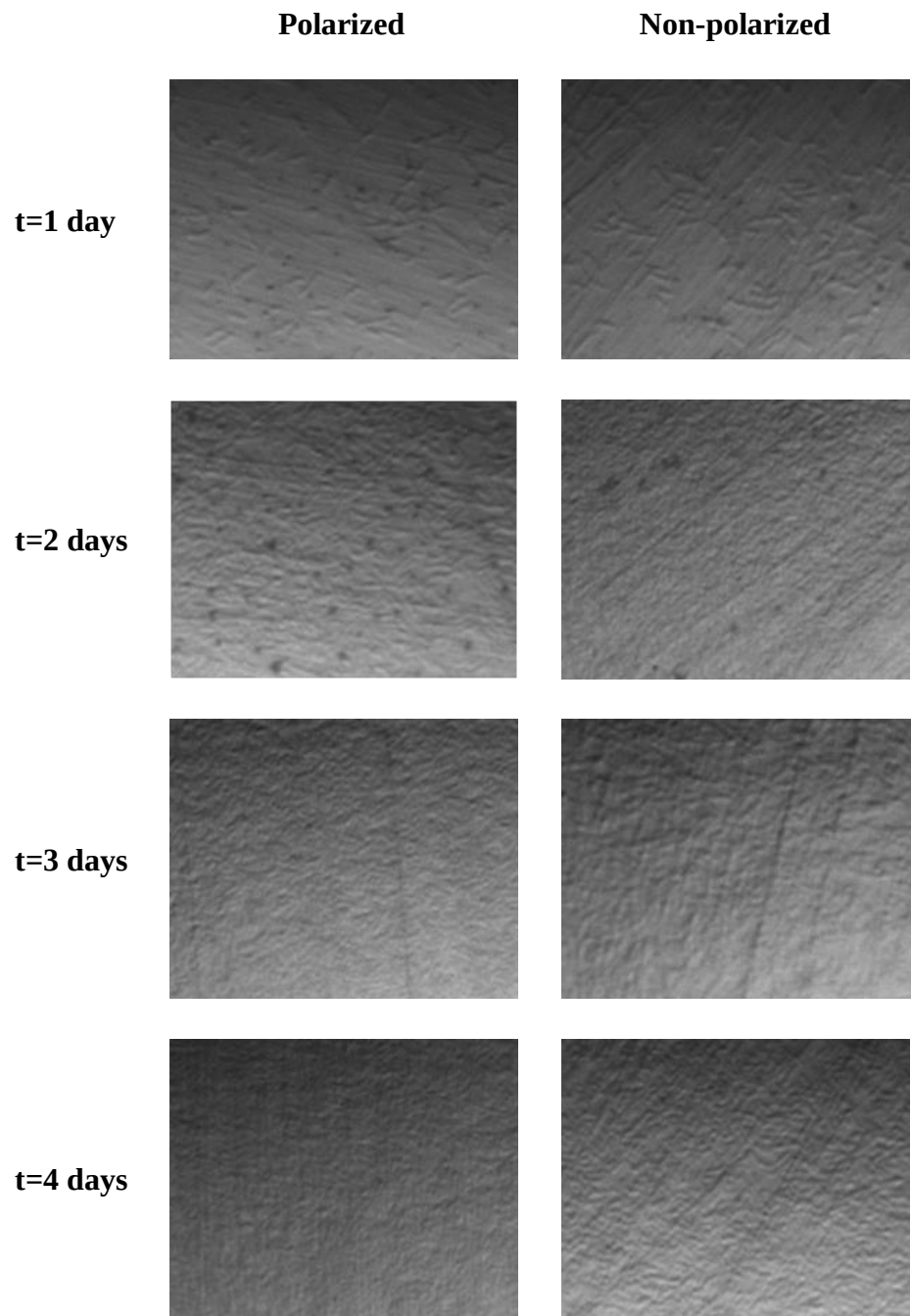


Figure 2. Representative images of the electrodes demonstrating cell attachment and growth over time.

Figure 2 quantifies how the number of cells increased by time for the polarized and non-polarized electrodes. The cell numbers were very small after 1 day (6×10^4 cells/cm² for polarized and 5×10^4 for non-polarized, there was no statistically significant difference between the two, t-test $P=0.27$). After day 1 the number of cells started to increase exponentially. During the experiment, the number of cells on the polarized and non-polarized electrodes were statistically similar ($P=0.12$) demonstrating that the cells grew similarly regardless of whether the electrode was polarized or not. This was unexpected. *S. oneidensis* can utilize lactate only if there is an electron acceptor available. For the polarized electrode, the electrode was the electron acceptor. However, for the non-polarized electrode, there was no electron acceptor directly provided. Possible explanations for this include 1) electrons were somehow deposited on the non-polarized surface, 2) small amounts of oxygen permeated into the system, or 3) endogenously produced electron acceptors were used, such as flavins or biomass. We know that non-polarized electrodes can build up charge, acting as a capacitor. If the bacteria are able to take advantage of this, we expect that the OCP of the non-polarized electrode will decrease with cell growth. However, it is unlikely that even an initially forming biofilm would be able to utilize this as a sole electron acceptor due to the amount of charge produced and the limited capacity of the electrode as a capacitor. To test these ideas, we measured open circuit potential of the non-polarized electrode and determined cell respiration as function of the inoculation density.

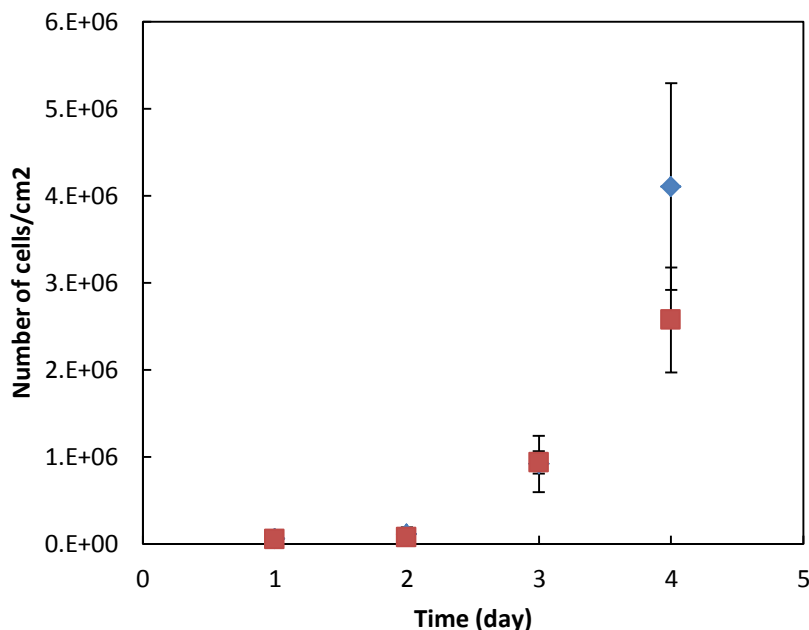


Figure 3. Number of cells on the polarized (◆) and non-polarized (■) surfaces (inoculation $OD_{600}=0.005$).

Figure 3 shows that the open circuit potential of the non-polarized electrode decreases with the cell growth and that its value depends on the inoculation density. When inoculation density was low, the variation of OCP with time was slow. For example, when the flow cell was inoculated with a culture with an optical density of 0.036, the OCP decreased from $\sim 200 \text{ mV}_{\text{Ag/AgCl}}$ to $50 \text{ mV}_{\text{Ag/AgCl}}$ within four days. The change in OCP was around 150 mV. However, when the flow cell was inoculated with higher cell density ($OD=0.301$), the OCP decreased from $\sim 300 \text{ mV}_{\text{Ag/AgCl}}$ to $\sim -180 \text{ mV}_{\text{Ag/AgCl}}$ within four days. This change of $\sim 480 \text{ mV}$ was almost three times higher than the change for low-cell density inoculum. Interestingly, the OCP value reached $\sim -180 \text{ mV}_{\text{Ag/AgCl}}$ within two days and remained constant, indicating that the OCP reached steady state.

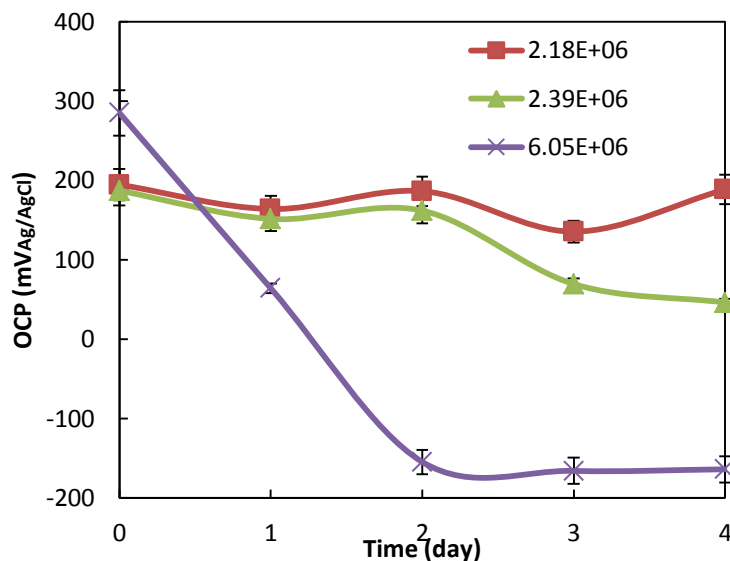


Figure 4. Open circuit potential (OCP) of the non-polarized electrode decreases with the cell growth and depends on the initial number of cells/cm².

Figure 4 shows cell respiration rates calculated as current per cell for the first day of cell attachment for different inoculation densities. We found that an inoculation density of 0.036 gave the maximum cell respiration rates, whereas higher inoculation densities decreased cell respiration rates. However, these rates leveled off (~ 8 fA/cell) for higher inoculation densities. Our results indicated that for the estimation of cell respiration rates, there must be a critical number of cells attached to the surface and for our system this number was $\sim 3 \times 10^6$ cells/cm².

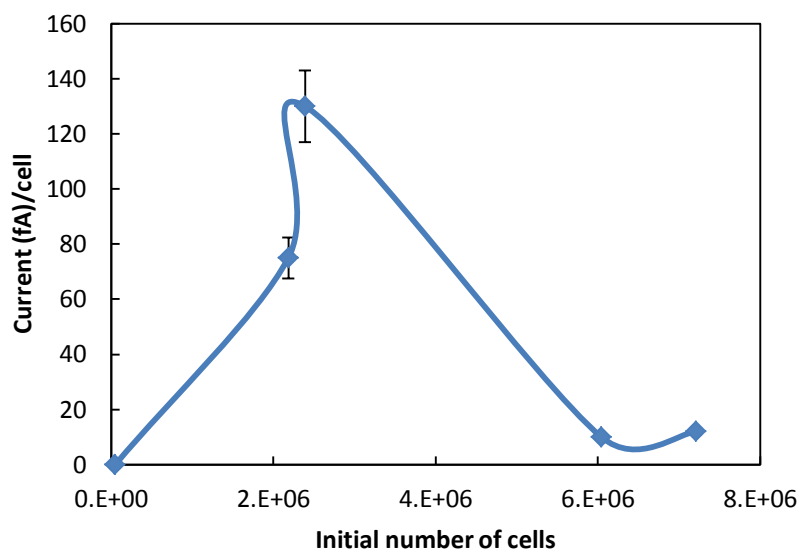


Figure 5. Initial number of cells versus cell respiration rate for 24h.

4. Practical implications

Our results demonstrated that *S. oneidensis* MR-1 cells can grow on electrodes that are not polarized, even under anaerobic conditions. After the cells are attached to the surface, the OCP of the electrode decreases as the cells grow. This decrease may be the result of happens because the electrons generated being deposited on the electrode surface, which acts as a capacitor, or more likely due to the change in concentration of redox couples near the electrode surface.

5. Conclusions

Growth of the cells on the polarized and non-polarized electrodes was statistically similar. Average cell respiration rate is critically dependent on the inoculum density. The denser the cell inoculation, the lesser is the average cell respiration.

The potential of the non-polarized electrode was dependent on the inoculation density.

Maximum cell reparation rate (130 fA/cell) was observed when initial number of cells was 3×10^6 cells/cm².

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CHAPTER 3

ELECTROCHEMICAL BIOFILM CONTROL: MECHANISM OF ACTION

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In this work, I conducted the bioelectrochemical flow cell experiments and imaging.

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Abstract

Although it has been previously demonstrated that an electrical current can be used to control biofilm growth on metal surfaces, the literature results are conflicting and there is no accepted mechanism of action. One of the suggested mechanisms is the production of hydrogen peroxide on metal surfaces. However, there are literature studies in which hydrogen peroxide could not be detected in the bulk solution. This is most likely because hydrogen peroxide was produced at a low concentration near the surface and could not be detected in the bulk solution. The goals of this research were 1) to develop a well-controlled system to explain the mechanism of action of the bioelectrochemical effect on 316L stainless steel (SS) surfaces, 2) to develop a technology to measure the hydrogen peroxide concentration near polarized SS surfaces, and 3) to test whether the produced hydrogen peroxide can reduce cell growth on metal surfaces. It was found that hydrogen peroxide was produced near 316L SS surfaces when a negative potential was applied. The hydrogen peroxide concentration increased towards the surface, while the dissolved oxygen decreased when the SS surface was polarized to $-600 \text{ mV}_{\text{Ag/AgCl}}$. When polarized and non-polarized surfaces with identical *Pseudomonas aeruginosa* PAO1 biofilms were continuously fed, the polarized surfaces showed ignorable biofilm growth while there was significant biofilm growth on the non-polarized surfaces. Although there was no detectable hydrogen peroxide in the bulk solution, it was found that the surface concentration of hydrogen peroxide was able to prevent biofilm growth.

Keywords: Biofilm, bioelectrochemical, biofilm control, hydrogen peroxide, cathode.

1. Introduction

Although electrical current has been previously demonstrated to control biofilm growth on metal surfaces, the literature results are conflicting and there is no single accepted mechanism of action (Del Pozo et al. 2008). Some of the mechanisms proposed in the literature are 1) electrochemical generation of oxidants on the surface (Costerton et al. 1994), 2) contraction or expansion of biofilm structure (Stoodley et al. 1997), 3) electrolytic generation of oxygen during water electrolysis (Stewart et al. 1999), and 4) electrostatic and electrophoretic repulsive forces (Hong et al. 2008). However, none of these mechanisms is widely accepted because of conflicting results in the literature and the impossibility of comparing the different electrochemical systems. This may be partly due to the oversimplification that the magnitude of the electrical current itself determines the effect on biofilm growth. Because our research group works with bioelectrochemical systems, information on current, potential, and internal resistances is needed to characterize the system, similar to microbial fuel cells (Logan et al. 2006). Thus, the term "bioelectrochemical effect" is used to describe the electrochemical control of biofilm growth on metal surfaces.

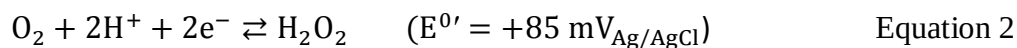
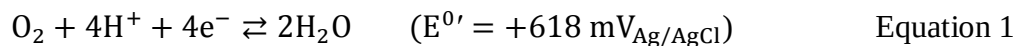
To control biofilm growth using the bioelectrochemical effect, the minimum electrochemical system that can be used is a two-electrode cell in which a current or potential is applied to the metal surface with biofilm against another electrode. Shirtliff et al. (2005) used an annular reactor with a stainless steel surface to evaluate the bioelectrochemical effect. They applied a $370\text{-}\mu\text{A}/\text{cm}^2$ current and found that the applied current did not have any bactericidal effect (Shirtliff et al. 2005). Similarly, Hong et al. (2008) used a flow chamber and applied a $15\text{-}\mu\text{A}/\text{cm}^2$ current and found that the applied current caused detachment of the biofilm on the

indium tin oxide surface (Hong et al. 2008). They hypothesized that this was due to electrostatic and electrophoretic repulsive forces. Similar observations were made by Van der Borden et al. (2004), who applied a current between 2.86 and 4.76 $\mu\text{A}/\text{cm}^2$ (van der Borden et al. 2004). Liu et al. (1997) inserted two electrodes into agar in a plate and found a zone where biofilm growth was inhibited (W. K. Liu et al. 1997). They found that this was due to hydrogen peroxide production. On the other hand, Del Pozo et al. (2009) applied a current between 1.626×10^3 and 1.626×10^6 $\mu\text{A}/\text{cm}^2$ and did not detect hydrogen peroxide; instead they concluded that pH was the cause of decreased biofilm growth (Del Pozo et al. 2009).

On the other hand, other researchers have controlled the potential of surfaces to prevent cell growth. For example, Busalmen and Sanchez (2001) poised a gold surface at negative potentials and observed a reduction in biofilm growth (Busalmen and de Sanchez 2001). They hypothesized that this was a result of electrostatic repulsion and pH changes caused by oxygen reduction. Similarly, Matsunaga and Lim (2000) poised nylon fishnets coated with conductive paint at negative potentials and observed biofilm detachment (Matsunaga and Lim 2000). Toxic substances such as hydrogen peroxide, oxidizing radicals, and chlorine molecules were not detected. Since our work focused on the bioelectrochemical effect only, the literature related to the combined use of antibiotics and electrical current known as the "bioelectric effect" is not discussed (Del Pozo et al. 2008). The use of combined antibiotics and electrical current could involve different mechanisms than those discussed in this paper.

It is important to note that the literature studies discussed above used different electrochemical cells with different geometries and operated using either a controlled potential—potentiostatic

system—or a controlled current—galvanostatic system—to achieve the bioelectrochemical effect. Regardless of the type of system, a specified current is required to understand the rate of reaction and a specified potential is required to assess the thermodynamic state of the system. To have reproducible or comparable data, both the potential (galvanostatic) and current (potentiostatic) data must be presented. Additionally, the potential of the electrode must be defined against a well-known reference electrode. Therefore it is critical to use a well-defined electrochemical system to understand the mechanisms of action of the bioelectrochemical effect. One mechanism of action of the bioelectrochemical effect has been suggested to be hydrogen peroxide generation. However, this has never been investigated near metal surfaces (Davis et al. 1994). According to Equation 1, oxygen is reduced to water on metal surfaces (Bard et al. 1985, De Schamphelaire et al. 2010, Freguia et al. 2007, Qiao et al. 2010). However, at the same time the partial reduction of oxygen leads to hydrogen peroxide production on metal surfaces (Equation 2) (Allen J. Bard 2001).



Although the standard reduction potential of hydrogen peroxide is +85 mV_{Ag/AgCl}, it is common to observe the onset of hydrogen peroxide production at negative polarization potentials because of large activation overpotentials (Vetter 1967). In bioelectrochemical systems where oxygen is

the only cathodic reactant, cathodic current will most likely result in the production of hydrogen peroxide.

By controlling the potential of the electrode, it is possible to generate hydrogen peroxide on 316L stainless steel (SS) surfaces. To the best of our knowledge the generation of hydrogen peroxide on SS surfaces has not been experimentally demonstrated to be a mechanism of the bioelectrochemical effect. The goals of this research were 1) to develop a well-controlled system to explain the mechanism of action of the bioelectrochemical effect on SS surfaces and 2) to test whether electrochemically generated hydrogen peroxide can reduce biofilm growth on metal surfaces. A flat plate flow cell was designed to monitor *in situ* biofilm formation. Previously developed hydrogen peroxide microelectrodes were modified to measure hydrogen peroxide on SS surfaces. In addition, dissolved oxygen (DO) microelectrodes were used to measure the variation of DO near the SS surface. Since hydrogen peroxide production was only found near the SS surface, its biocidal efficacy on *Pseudomonas aeruginosa* PAO1 biofilms was tested. *P. aeruginosa* PAO1 biofilms were grown on SS surfaces and biofilm growth was quantified on polarized and non-polarized surfaces.

2. Materials and methods

2.1. Preparation of growth medium and inoculum

P. aeruginosa PAO1 tagged with green fluorescent protein was obtained from Dr. Michael Franklin, Center for Biofilm Engineering, Montana State University. A defined medium with the following composition was used: Na₂HPO₄, 1830 mg l⁻¹ (J.T. Baker®); KH₂PO₄, 1350 mg l⁻¹

(J.T. Baker ®); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg l^{-1} (J.T. Baker®); yeast extract, 10 mg l^{-1} (Bacto®); glucose, 1000 mg l^{-1} (Fisher®); $(\text{NH}_4)_2\text{SO}_4$, 100 mg l^{-1} (J.T. Baker®). The medium was autoclaved at 121°C for 15 min per liter of solution. The ammonium sulfate and yeast extract were autoclaved separately and then added to the growth medium. The glucose was filter sterilized. After the medium was allowed to cool to room temperature, a trace elements solution (1 ml l^{-1}) was filtered and added to it. The trace elements solution was prepared by adding $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 357 mg l^{-1} ; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 228 mg l^{-1} ; $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 317 mg l^{-1} ; $(\text{NH}_4)\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$, 231 mg l^{-1} ; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 127 mg l^{-1} ; ZnCl_2 , 363 mg l^{-1} ; FeCl_3 , 2160 mg l^{-1} ; and CaCl_2 , 3700 mg l^{-1} . Carbenicillin (150 $\mu\text{g ml}^{-1}$ of USP Grade disodium salt, Teknova) was added to the medium. A 1-ml vial of frozen (-85°C) *P. aeruginosa* PAO1 stock was thawed at room temperature. The stock was then used to inoculate 100 ml of the sterilized defined medium, which was incubated on a shaker at 150 rpm at 30°C for 24 h. The optical density (OD) was measured at 600 nm and adjusted to 0.05 before the biofilm reactor was inoculated. An OD of 0.05 was chosen as optimal for obtaining a single layer of cells during initial attachment and quantifiable cell counts on the SS surface subsequently. The flat plate flow cell was inoculated with 10 ml of inoculum using a sterile syringe.

2.2. Preparing the biofilm reactor

The experimental setup is shown in Figure 1. A custom-made flat plate flow cell was used to grow biofilms on SS surfaces. The reactor was designed in such a way that the SS working electrode and the graphite counter electrode (Glassmate grade GM-10, Poco Graphite, Inc., Decatur, TX, USA) were separated by a cation exchange membrane (Membranes International Inc., CMI-7000, 6×8 cm). The membrane isolated the reactions at the counter electrode from the

SS working electrode. The reactor bottom was a viewing window for epifluorescence microscopy made of microscope cover glass (Thermo Scientific® Gold Seal Cover Glasses, Rectangles 48×60 mm, #1). In this way, the cells on the SS surface were imaged directly. Five autoclavable rubber separators were used between each two layers to prevent leakage. A graphite (1×2 cm) counter electrode and a custom-made Ag/AgCl reference electrode (Lewandowski and Beyenal 2007) were used in the upper compartment. Two SS coupons (1.18×1.18 cm) were used in the lower cells. The first SS coupon was the control surface (non-polarized) and the second SS coupon was polarized against the Ag/AgCl reference electrode. Platinum wires (CA Fine Wire Company, Pure TC Grade, MO, #M159850) were used to connect the SS coupons to external cables which were connected to a custom-built potentiostat (Renslow et al. 2011). The connection resistances were always less than 5 Ω .

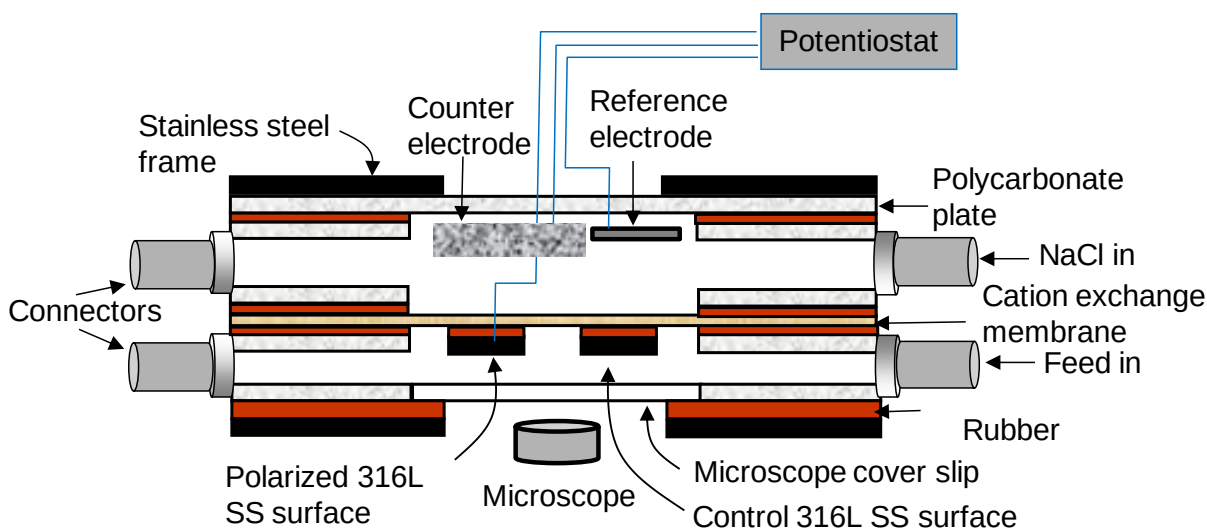


Figure 1. Schematic configuration of experimental setup and flat plate flow cell. The figure is not drawn to scale.

2.3. Operating the biofilm reactor

The assembled flat plate flow cell (Figure 1) and tubing were autoclaved at 121°C for 15 min. After cooling to room temperature, the 316L SS working, the counter and the reference electrodes were connected to a custom-built potentiostat (Renslow et al. 2011). A two-channel peristaltic pump (Masterflex[®] L/S[®] Cole-Parmer Instrument Company) was used at a flow rate of 0.4 ml/min for the feed and 3M NaCl solution. The flow cell was inoculated aseptically from its inoculation port with 10 ml of inoculum. After 2 h of initial attachment (no flow), the flow was started and planktonic cells were washed out of the flow cell. Before polarization, it was verified that there were no bubbles in the reactor. For biofilm experiments, the polarization potential was set to -600 mV_{Ag/AgCl}.

2.4. Biofilm imaging and analysis

During operation, the flat plate flow cell was placed on an inverted epifluorescence microscope (Nikon[®] Eclipse Ti-S inverted microscope); a Nikon[®] DS-Qi1Mc camera was mounted on the microscope for imaging. A CFI Plan Fluor ELWD 40× objective (N.A. 0.60, W.D. 3.7- 2.7 mm) was used to image the cells. The images were taken every eight hours. At least 20 images were taken to obtain statistically representative data (Ica et al. 2012). The surface coverage was calculated from these images using NIS[®] software. Surface coverage is the ratio of the area of biomass to the total area of the image (NIS-Elements Users Guide 2010). The higher the surface coverage, the higher the coverage of the surface by biomass.

2.5. Scanning electron microscopy

After 40 h of treatment, both the polarized and the non-polarized 316L SS surfaces were removed aseptically from the flat plate flow cell. The SS coupons were incubated overnight with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer followed by rinsing with 0.1 M phosphate buffer (3×10 mins each). SS coupons were dehydrated gradually using 30%, 50%, 70%, and 95% alcohol (10 mins each) and 100% alcohol (3×10 mins each). Hexamethyldisilazane (HMDS) was used for overnight drying. The SS coupons were sputter-coated with gold prior to FEISEM (FEI 200F) imaging.

2.6. Microelectrode construction

The hydrogen peroxide and oxygen microelectrodes were constructed following the procedures given in Lewandowski and Beyenal (2007) except that the hydrogen peroxide microelectrode was modified so that it could operate on polarized surfaces (Lewandowski and Beyenal 2007). Briefly, an etched platinum wire was encased in a glass capillary which was pulled at a high temperature to seal the wire. The platinum tip was then exposed by grinding away the sealed tip using a diamond grinding wheel (Narishige®, model EG-4). The exposed tip was 10-15 µm in diameter. Platinum was electrochemically deposited onto the exposed platinum tip, which resulted in a porous platinum ball that increased the effective surface area and consequently the sensitivity of the microelectrode. A 5% cellulose acetate (Sigma-Aldrich®) membrane was then deposited on the platinum ball. The final tip diameter was ~20 µm. The hydrogen peroxide microelectrode was placed in a glass outer casing with the tip exposed to the solution. The glass junction was sealed with an agar (1.812-g l⁻¹ R-2A Agar, Fluka®) salt bridge containing 1 mM sodium sulfate (Fisher®) and the outer case was filled with saturated KCl and Ag/AgCl

reference filling solution (SP135-500, Fisher®). A Ag/AgCl reference electrode was inserted into the glass outer casing. The procedures given in the literature were followed to construct the dissolved oxygen microelectrode (Lewandowski and Beyenal 2007). The final tip diameter of the DO microelectrode was ~15 μm .

2.7. Operation of microelectrodes

The hydrogen peroxide and oxygen microelectrodes are amperometric sensors. A Keithley® 6517A Electrometer/High Resistance Meter was used as both the voltage source and a picoammeter. The hydrogen peroxide microelectrode was polarized to +600 $\text{mV}_{\text{Ag/AgCl}}$ and the DO microelectrode was polarized to -800 $\text{mV}_{\text{Ag/AgCl}}$. The hydrogen peroxide microelectrode was calibrated in solutions of known hydrogen peroxide concentration ranging from 0 μM to 150 μM . The calibration curve was always linear in this concentration range. The oxygen microelectrode was calibrated in a solution with zero oxygen concentration and in air-saturated sterile growth medium. Each microelectrode was calibrated right before use and the calibrations were also verified after the measurements.

2.8. Reactor used for microelectrode measurements

Since the reactor shown in Figure 1 did not have a port for microelectrode measurements, a flat plate reactor with recycle was used (Nguyen et al. 2012). The SS coupons were placed at the bottom of this reactor so that it was possible to measure DO or hydrogen peroxide profiles on the SS surface. For control experiments, a clean SS was used. For experiments with biofilms, *P. aeruginosa* PAO1 cells were allowed to attach to a SS surface using the procedure described

above. After initial attachment, the growth medium was replaced several times to remove suspended cells. Air-saturated growth medium was continuously fed into the reactor.

Microelectrode measurements with biofilm were conducted in a biosafety level II hood.

2.9. Microelectrode measurements

To determine the polarization potential at which hydrogen peroxide was produced at the SS surface, the hydrogen peroxide microelectrode was positioned approximately 100 μm from the SS surface and the potential was scanned from +200 $\text{mV}_{\text{Ag/AgCl}}$ to -650 $\text{mV}_{\text{Ag/AgCl}}$. The hydrogen peroxide concentration was monitored as a function of the potential of the SS. A distance of 100 μm from the SS surface was maintained to reduce the likelihood of breaking the microelectrode tip. The current values during the potential sweep were collected automatically by the potentiostat.

To measure depth profiles of hydrogen peroxide or DO, the tip of the microelectrode was positioned approximately 800 μm above the SS surface using micromanipulators (Narishige®). The microelectrode tip was then stepped down in 5- μm increments using custom microprofiler software until the tip was 100 μm from the SS surface. Concentrations were measured at each step. The measurement of profiles was stopped 100 μm from the SS surface to ensure that the tip of the microelectrode was not broken for repeated measurements. Each measurement was repeated at least three times in total. The locations of the microelectrode tip and the SS surface were monitored using a stereomicroscope (Zeiss® Stemi 2000 stereomicroscope). A Gamry® Series GTM 300 potentiostat. (Gamry Instruments, Warminster, PA, USA) was used to polarize

the SS coupon to $-600 \text{ mV}_{\text{Ag/AgCl}}$ during depth profile measurements. Subsequently, depth profiles were taken in the same position under non-polarized and polarized conditions by controlling the potentiostat. A graphite rod counter electrode (10 cm in length and 0.5 cm in diameter) and a Ag/AgCl reference electrode were used. Note that the current was allowed to stabilize before each depth profile was measured.

To find the SS surface the tip of the microelectrode had to be broken by touching the surface, which did not allow us replications. Therefore, depth profile measurements were stopped before the microelectrode tip touched the surface, except during the last measurement, when the tip was broken to determine the location of the SS surface. It was found that the profiles near the SS were always linear.

3. Results and discussions

The microelectrode measurements were conducted twice (two replicates) and repeated at least three times at each location. The biofilm experiments were conducted in replicates and repeated at three different times. The results were all statistically similar and led to identical conclusions. Here selected representative measurements are shown.

3.1. Quantifying hydrogen peroxide generation and oxygen consumption at the 316L stainless steel surface

Figure 2 shows the variation of hydrogen peroxide concentration with potential at a position $100 \mu\text{m}$ from the SS surface. During the potential scan between $+200 \text{ mV}_{\text{Ag/AgCl}}$

and $-650 \text{ mV}_{\text{Ag/AgCl}}$, the hydrogen peroxide concentration reached a transient concentration of $5.9 \text{ }\mu\text{M}$. The onset of detectable hydrogen peroxide at the microelectrode tip was at approximately $-400 \text{ mV}_{\text{Ag/AgCl}}$, although it was expected to correspond with the onset of cathodic current at $-200 \text{ mV}_{\text{Ag/AgCl}}$. The delay in detection of hydrogen peroxide at the microelectrode tip can be attributed to a combination of diffusion, catalytic decomposition of hydrogen peroxide, and further reduction of hydrogen peroxide to water. According to Vetter (1967), oxygen reduction at metal electrodes proceeds initially with Equation 2 (Vetter 1967). At more negative potentials, hydrogen peroxide is further reduced to water, which results in the complete reduction of oxygen to water as shown in Equation 1 (Vetter 1967). On iron electrodes, the transition from Equation 2 to Equation 1 occurs between $-400 \text{ mV}_{\text{Ag/AgCl}}$ and $-800 \text{ mV}_{\text{Ag/AgCl}}$ (Vetter 1967). Beyond $-800 \text{ mV}_{\text{Ag/AgCl}}$, Equation 1 dominates and hydrogen peroxide production is minimal. Figure 2 shows the onset of the transition at $-400 \text{ mV}_{\text{Ag/AgCl}}$ and its continuation through $-650 \text{ mV}_{\text{Ag/AgCl}}$. The observation of a potential range for hydrogen peroxide production provides a key point for the bioelectrochemical effect. The production of hydrogen peroxide cannot be understood by considering current values alone. The limitation of reporting only the current values could explain the conflicting evidence on the role of hydrogen peroxide in inhibiting biofilm growth (Busalmen and de Sanchez 2001, Costerton et al. 1994, Hong et al. 2008, Shirtliff et al. 2005). At sufficiently negative potentials, hydrogen peroxide could go undetected by methods sampling the bulk solution.

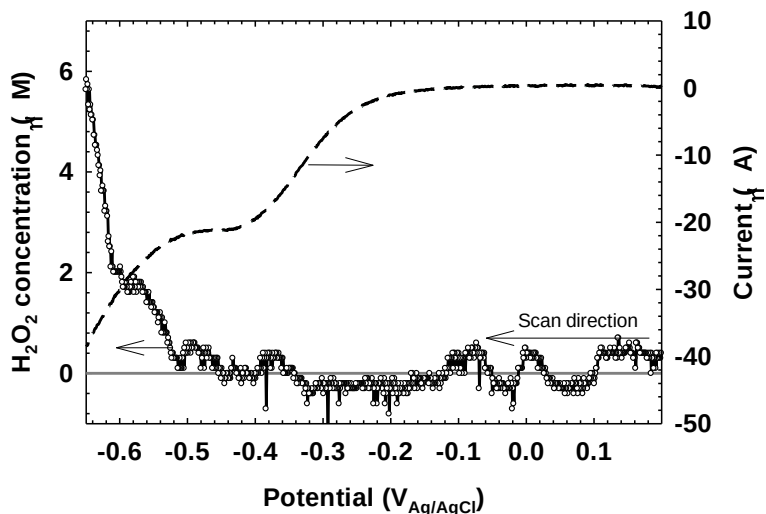


Figure 2. Transient hydrogen peroxide concentration variation with potential 100 μm from the SS surface. The black solid line represents the zero concentration line. The dashed line is the current measured during the potential sweep.

Figure 3A shows the hydrogen peroxide profiles that were measured above the SS surface, from the bulk to within 100 μm of the surface. The hydrogen peroxide concentration was 15.5 μM near the SS surface at a potential of -600 $\text{mV}_{\text{Ag/AgCl}}$. When the SS was not polarized, there was no detectable hydrogen peroxide. However, Figure 3A shows that hydrogen peroxide was also not detectable at a distance of 500 μm from the SS surface while it was polarized to -600 $\text{mV}_{\text{Ag/AgCl}}$. Considering that hydrogen peroxide degrades in water and that the flow cell was operated in continuous mode, hydrogen peroxide was unlikely to be found in the bulk solution. This result highlights the limitation of hydrogen peroxide measurements using bulk sampling methods in this type of system. However, it also emphasizes the suitability of the system for preventing biofilm growth near the surface. We expect that attached cells on the SS surface are

exposed to hydrogen peroxide concentrations of at least 15.5 μM . The concentration of hydrogen peroxide could be enough to prevent a monolayer cell coverage ($\sim 1\ \mu\text{m}$ thick) from growing into a mature biofilm.

Figure 3B shows the oxygen profiles that were measured above the SS, from the bulk to within 100 μm of the surface. The oxygen concentration decreased to $\sim 3.5\ \text{mg l}^{-1}$ near the SS surface from a bulk concentration of $\sim 7.0\ \text{mg l}^{-1}$ when the SS was polarized to $-600\ \text{mV}_{\text{Ag/AgCl}}$. When the SS was not polarized, there was a slight decrease in oxygen concentration, suggesting that cells were utilizing oxygen to respire. The consumption of oxygen while a cathodic current was measured confirms oxygen reduction as the source of hydrogen peroxide. The reduced DO at the SS surface also shows that the production of hydrogen peroxide at the surface drives the environment from an oxic to a suboxic condition. Thus, if hydrogen peroxide exposure is the primary mechanism of action of the bioelectrochemical effect, a simultaneous decrease in oxygen may also play a role. Since one of the proposed mechanisms of action in the literature is pH changes caused by the passing of current, we also quantified pH variation: there were no detectable pH changes near the SS surface (results not shown). This was expected, since the measured current was too low for the detection of significant pH changes at this buffer strength (Babauta et al. 2011).

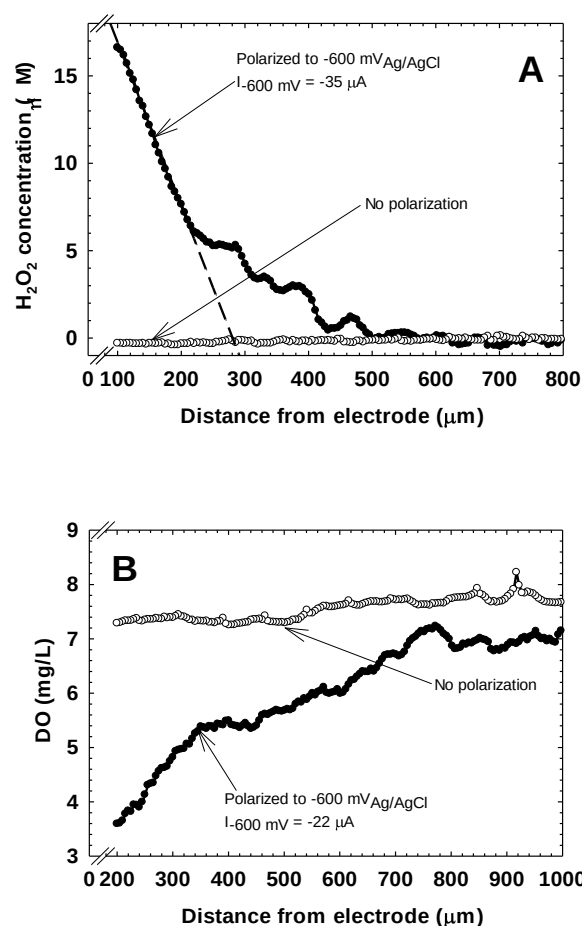


Figure 3. A) Hydrogen peroxide profiles above both non-polarized SS surfaces ($\circ$) and polarized SS surfaces ($\bullet$). The black dashed line is fitted to points between 100 and 215 μm ($R^2=0.997$). B) Oxygen profiles above both non-polarized SS surfaces ($\circ$) and polarized SS surfaces ($\bullet$). The current density was 25.14 $\mu\text{A}/\text{cm}^2$.

One of the major limitations of this method of controlling biofilm growth is the requirement that oxygen be present at the SS surface. If a thick biofilm initially covered the SS surface, the DO concentration would be expected to be zero near the bottom of the biofilm, which would prevent hydrogen peroxide production (Renslow et al. 2011). Perhaps this too is a reason for the

conflicting data in the literature. The method could be optimized for thicker biofilms by increasing the delivery of oxygen to the surface, but the added energy input would still make this method less efficient for thicker biofilms. Since our biofilm was a single layer of cells, there was oxygen available on the surface. Using these thin biofilms, it was possible to assess the effect of hydrogen peroxide production on biofilm growth.

3.2. Biofilm formation on the non-polarized and polarized SS coupons

To assess the effect of the hydrogen peroxide generated by applying a potential of $-600 \text{ mV}_{\text{Ag/AgCl}}$, a monolayer of cells was exposed for 40 hours, as shown in Figure 4. The initial cell surface coverage was approximately $1.9 \pm 0.5\%$ for both non-polarized and polarized SS surfaces. There was no statistical difference in surface coverage by biofilms initially ($P=0.808$). The surface coverage increased on the non-polarized SS surface to $99 \pm 0.6\%$ after 40 hours, whereas the surface coverage remained almost constant for the polarized SS surface. The differences between the non-polarized and polarized SS surfaces were statistically significant ($P = <0.001$). It was clear that uncontrolled cell growth on the non-polarized SS surfaces resulted in large biofilm clusters. In contrast, dim patches of light intensity behind a sparsely populated SS surface characterized the polarized SS surface. No small or large biofilm clusters were observable, signifying that biofilm growth was delayed. In a similar experiment, initially attached cells were treated with $50 \text{ }\mu\text{M}$ hydrogen peroxide in the bulk solution and the surface coverage was monitored. Nearly identical results were observed for externally added hydrogen peroxide and electrochemically produced hydrogen peroxide (data not shown). The addition of hydrogen peroxide to the bulk solution resulted in delaying biofilm growth. Interestingly, this

suggests that the decrease in DO during oxygen reduction at the SS surface played only a minor role in delaying biofilm growth.

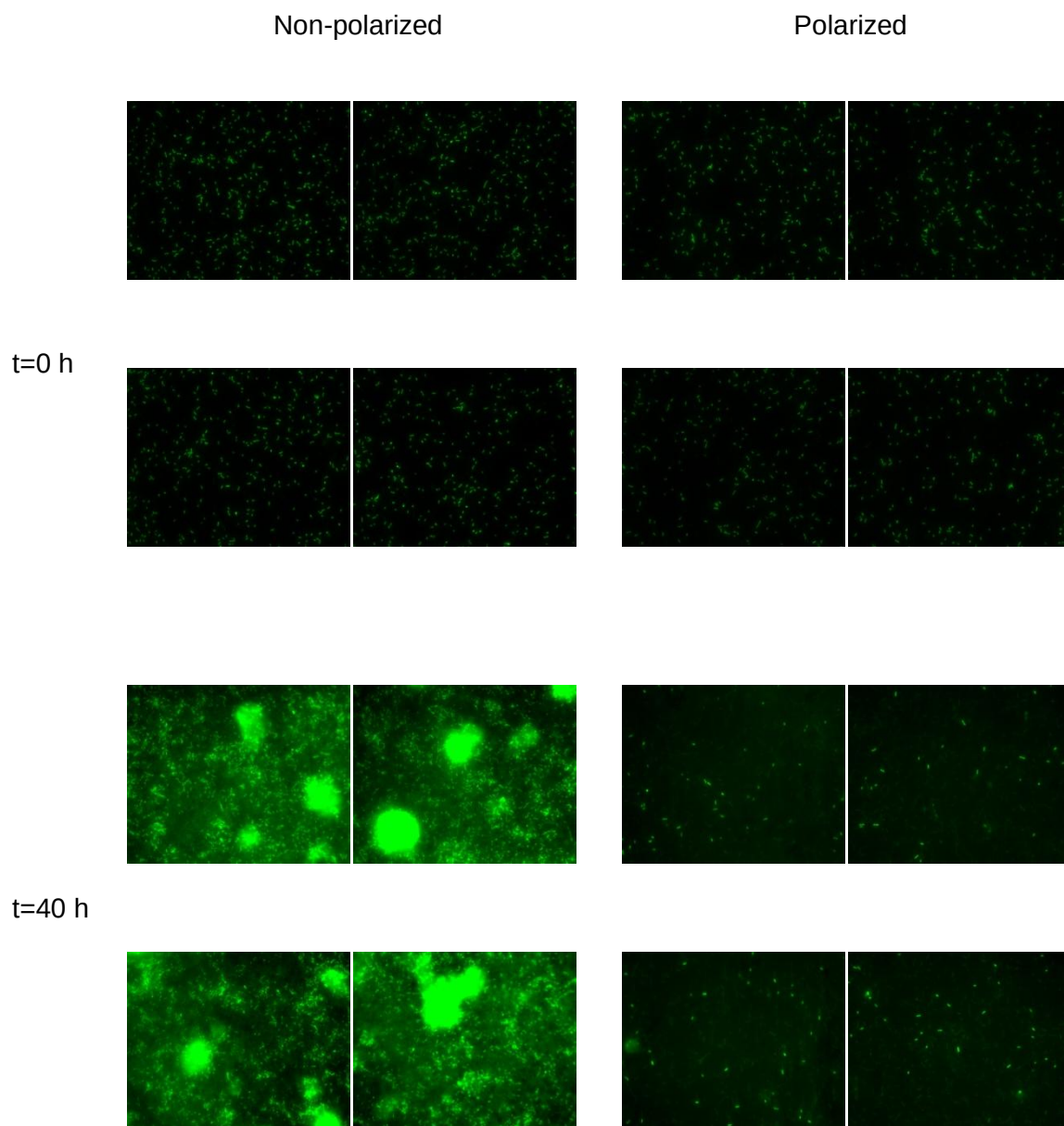


Figure 4. Images of *P. aeruginosa* PAO1 on non-polarized and polarized surfaces at t=0 and t=40 h (four images shown for each condition). The initial surface coverages were identical (1.9 ± 0.5). However, the final surface coverages were statistically different ($P = < 0.001$). The image size is $204.8 \mu\text{m} \times 163.83 \mu\text{m}$.

Figure 5A plots this time series and shows the variation with time of surface coverage of the SS surface. The non-polarized SS surface showed an exponential increase in surface coverage similar to the exponential growth measured by optical density. The polarized SS surface showed no measurable increase in surface coverage over time, which demonstrates the effectiveness of polarization in controlling biofilm growth. Over the length of exposure, the current reached a steady state value of $\sim 60 \mu\text{A}$. Thus, it is thought that the monolayer of cells was exposed to at least $15.5 \mu\text{M}$ hydrogen peroxide under the polarized condition and a subsequent decrease in DO. Although seemingly small, these hydrogen peroxide concentrations have been shown to cause DNA damage (Linley et al. 2012).

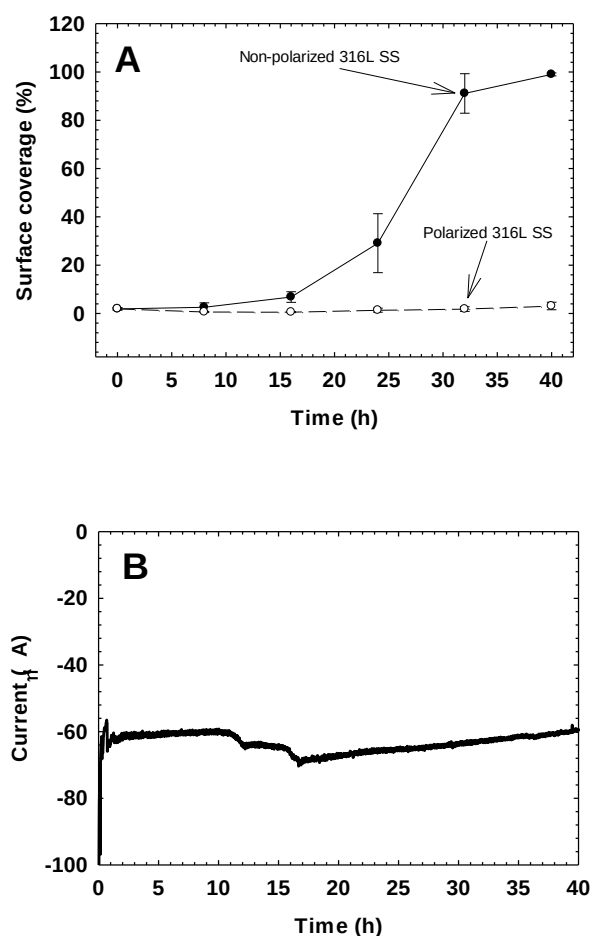


Figure 5. A) The variation in surface coverage of polarized ($\circ$) and non-polarized ($\bullet$) 316L SS surfaces over time. B) The variation in current over time for the polarized 316L SS.

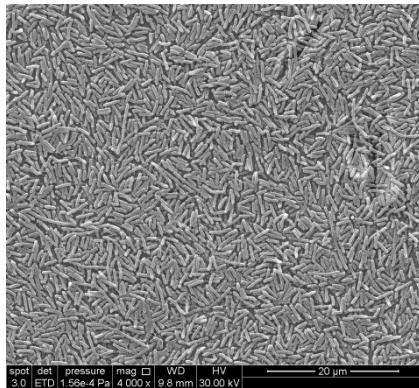
3.3. SEM analysis of biofilm growth on SS surfaces

To confirm the images presented in Figure 4, SEM images were taken of the SS coupons after fluorescent imaging. The SEM images in Figure 6 show that the non-polarized surface was covered with cells (Figure 6A) while fewer cells colonized the polarized surface (Figure 6B). Higher magnification of individual cells on the non-polarized SS surface showed intact cell walls (Figure 6C). On the polarized SS surface, membrane-compromised cells surrounded by scattered

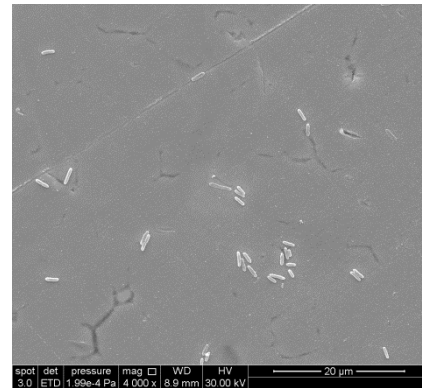
deposits of material were observed (Figure 6D). It has been shown that cell walls and cell membranes of *P. aeruginosa* 19142 are deformed when treated with hydrogen peroxide (DeQueiroz and Day 2007). This is because hydrogen peroxide targets thiol groups of enzyme on the cell wall (Denyer and Stewart 1998). Intracellular contents are lost and, as a result, cell volume decreases (Linley et al. 2012). Similar images were also obtained by Diao *et al.* when cells were treated with hydrogen peroxide (Diao et al. 2004). The scattered deposits observed in Figure 6D were identified as cell debris. These two observations provide further evidence that the electrochemical production of hydrogen peroxide at the SS surface was the cause of the delayed biofilm growth.

Non-polarized

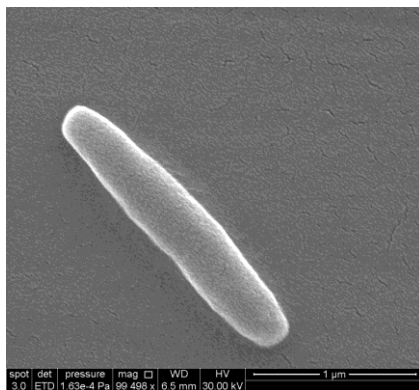
Polarized



A



B



C



D

Figure 6. SEM images of cells on non-polarized (A and C) and polarized coupons (B and D).

Figure 4 and Figure 6B showed that despite the 40-hour polarization treatment, some cells were able to survive and persist on the polarized SS surface. This could be the result of heterogeneous current distribution on the SS surface, increased catalase production by these surviving cells, or a combination of both. Since these cells expressed GFP, they were most likely alive as demonstrated in the literature (Nancharaiah et al. 2005). Interestingly, catalase actively

degrading hydrogen peroxide could be another reason for hydrogen peroxide not being detected in the bulk solution. This possibly explains why some of the studies in the literature found hydrogen peroxide while others did not. Regardless of the mechanism that these cells used to survive, the bioelectrochemical effect did not kill all the cells on the polarized SS surfaces observed in this study. Thus, it is important to note that, although hydrogen peroxide has a biocidal effect on cells, the bioelectrochemical effect as a whole should be considered biostatic rather than biocidal.

4. Practical implications

In this research, it has been demonstrated that hydrogen peroxide is produced on SS surfaces and that it can be used to prevent biofilm growth. However, for hydrogen peroxide production, the biofilm must be thin and allow oxygen penetration to the bottom of the biofilm. Hydrogen peroxide cannot be produced when there is no DO on the SS surface. Thus, the thickness of the biofilm could be a critical factor when controlling biofilm growth electrochemically. Perhaps the best method of preventing cell growth on SS surfaces is to keep the potential of the surface at -600 mV_{Ag/AgCl} as soon as the process starts. Continuous hydrogen peroxide production may delay biofilm growth but may not eliminate long-term biofilm growth. Fewer cells might colonize but be able to survive and persist. Although we used a catalase-positive bacterium that can actively decompose hydrogen peroxide, electrochemically produced hydrogen peroxide showed the biocidal effect. This technique could be more effective on catalase-negative bacteria. The presence of catalase could be critical in some systems if the biofilm producing catalase decomposes the produced hydrogen peroxide to oxygen. This could potentially prevent the

biocidal effect of hydrogen peroxide, as demonstrated by others (X. F. Liu et al. 1998, Stewart et al. 2000).

5. Conclusions

In this work hydrogen peroxide production and dissolved oxygen consumption were quantified on polarized 316L stainless steel surfaces using microelectrodes. The produced hydrogen peroxide was used to control *P. aeruginosa* PAO1 biofilm growth on SS surfaces. The following conclusions have been reached:

Hydrogen peroxide is produced at potentials below $-400 \text{ mV}_{\text{Ag/AgCl}}$ on SS surfaces. When the SS surface is polarized to $-600 \text{ mV}_{\text{Ag/AgCl}}$, hydrogen peroxide concentration is increased towards the surface while dissolved oxygen concentration is decreased. There was no hydrogen peroxide production or dissolved oxygen consumption on non-polarized clean surfaces. When polarized and non-polarized surfaces with identical biofilms continued to be fed with fresh medium, polarization prevented biofilm growth while there was significant biofilm growth on non-polarized surfaces. There was cell debris deposited on the polarized surfaces while there was no cell debris on non-polarized surfaces, indicating the biocidal effect of hydrogen peroxide.

Overall, hydrogen peroxide produced on SS surface can be used to control biofilm growth. However, this requires a continuous flux of dissolved oxygen to the surface.

Acknowledgements

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CHAPTER 4

ANTIBIOTIC MASS TO COLONY FORMING UNIT RATIO DETERMINES EFFECTIVENESS OF AN ANTIBIOTIC AGAINST BIOFILMS AND PLANKTONIC CELLS

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In this work, I developed the hollow fiber biofilm reactor and conducted the related experiments.

Abstract

This study tests the hypothesis that “biofilms are more resistant to antibiotics because the molar concentration of antibiotics per cell is less compared to planktonic cells when both planktonic and biofilms cell were treated with the same concentration of antibiotics.” To test this hypothesis, we used *Pseudomonas aeruginosa* PAO1 and challenged it with tobramycin as the model antibiotic. The cells were grown in a chemostat operated at steady state, and planktonic tests were performed using different cell densities. The cell densities were adjusted to a desired value by centrifuging and increasing their density. Alternatively, the biofilms were grown in a hollow fiber biofilm reactor. The hollow fiber biofilm reactor allowed us to extend our hypothesis to test biofilms cells by exposing the antibiotics at different amounts simply by eliminating mass transfer limitations. The planktonic and biofilm samples were tested with different tobramycin concentrations. We found that when *P. aeruginosa* PAO1 was challenged with tobramycin, there are three distinct zones in the response curves: 1) a low tobramycin concentration where there is no significant log reduction, 2) a linear effect zone where the log reduction increases linearly with the logarithm of the amount of tobramycin to total CFU in the sample, and 3) a concentration zone where tobramycin concentration controls the log reduction. When biofilm samples were challenged with tobramycin using different delivery strategies, we found the response critically depended on the concentrations. For low and high concentrations we did not see any differences between tobramycin delivered using different mechanisms. However, for a tobramycin concentration of 100 µg/mL we observed three distinct responses indicating the differences in tobramycin delivery. Our results demonstrated that in addition to well-known mechanisms of antibiotic resistance, the amount of antibiotic to the total number of cells should be considered.

Keywords: Biofilm, *Pseudomonas aeruginosa*, tobramycin, biofilm control, and antibiotic resistance.

1. Introduction

Antibiotic resistance in biofilms can be attributed to one or more of the following factors: slow or incomplete penetration of antibiotic into the biofilm (Stewart, 1996) , development of a resistant phenotype (Fujita et al., 1992), adaptive responses (Stewart and Costerton, 2001), quorum sensing (Hoiby et al., 2010), genetic transfer, expression of resistance genes (Brooun et al., 2000) , and persisters (Lewis, 2007). The extracellular matrix of biofilm also plays a critical role. It has the ability to impede the penetration of antibiotics either by slowing their diffusion or by reacting with them. When a biofilm matures, the extracellular polymeric substance deposition increases, leading to an increase in chemical gradients through the biofilm. In addition, consumption of oxygen near the surface of the biofilms develops anaerobic conditions inside of the biofilm (Lewandowski and Beyenal, 2007; Walters et al., 2003). The oxygen concentration gradient leads to a development of cells with higher metabolic activity on top of the biofilm and cells with low metabolic activity on the bottom (Harmsen et al., 2010). This causes reduction in the growth rate of biofilm cells that results in differentiation into non dividing cells (Stewart and Costerton, 2001). In *Pseudomonas aeruginosa* biofilms, the antibiotics interfering with processes such as replication (ciprofloxacin) and translation (tobramycin, gentamicin) specifically kill the cells on the surface of the biofilm without affecting the bottom layers (Harmsen et al., 2010). Bacteria in a biofilm can produce an autoinduction signaling molecule that accumulates in the environment when bacterial concentrations reach a critical number. Bacteria sense this high cell density and respond by activating genes that produce virulence factors like enzymes and toxins which function as protective against antimicrobial agents (Fux et al., 2005; Hoiby et al., 2010; Simoes et al., 2010). In addition, quorum sensing plays a major role in bacterial activities like production of exopolysaccharide, development of biofilm, and virulence (Simoes et al., 2010).

Bacteria in biofilms have the ability to exhibit stress responses when encountering environmental challenges such as temperature change, DNA damage, oxidative stress, etc. In case of biofilms, bacteria undergo gene mutation when exposed to antibiotics (Pace, 2005; Stewart, 2002). The mutations in *P. aeruginosa* are reported to be in the methyl-directed mismatch repair genes that are required for DNA repair and DNA oxidative lesions repair (Hoiby et al., 2010; Pace, 2005). The oxidative stress which is caused by production of reactive oxygen species leads to hypermutation in biofilms (Hoiby et al., 2010). Moreover, antibiotic resistance can be acquired through horizontal gene transfer between bacteria (Pace, 2005) via conjugation. Genetic transfer in biofilms is higher than that in planktonic cells due to close contact of bacteria in biofilms (Foley and Gilbert, 1996; Pace, 2005). Expression of genes that contribute to antibiotic resistance is one of the defense mechanisms in biofilms. Production of β lactamases, aminoglycoside inactivating enzymes, and overexpression of multidrug efflux pumps are some additional mechanisms responsible for antibiotic resistance (Haddadin et al., 2010; Hoiby et al., 2010). β lactamases are enzymes inactivating β lactam antibiotics and are expressed by overproduction of gene AmpC (Hoiby et al., 2010). Similarly, overexpression of multidrug efflux pumps causes greater efflux of antibiotic out of the membrane, thus hindering the intracellular action of antibiotic.

Some cells in a biofilm are resistant to antibiotic treatment and develop as slow growing cells called persisters (Keren et al., 2004; Lewis, 2007; Mulcahy et al., 2010). When the antibiotic concentration decreases, they resume the growth and repopulate the biofilm. These persisters are also protected from host defenses by the extracellular matrix (Lewis, 2007).

In summary, all of these response mechanisms can protect biofilms against antibiotics. However, it is often overlooked that the biofilm cells are denser than planktonic cells. The cell densities of planktonic cells are generally 1-5 g/L while biofilm densities are around 50 – 200 g/L (Lewandowski and Beyenal, 2007). Lewandowski (Lewandowski, 1985) noticed this and tested toxicity of heavy metals to biofilms and planktonic cells. He found that one of the reasons for resistance of the biofilms to toxicity was due to their high cell density. Based on these findings we hypothesize that biofilms are more resistant against antibiotics in the same way. This hypothesis can be visualized in Figure 1.

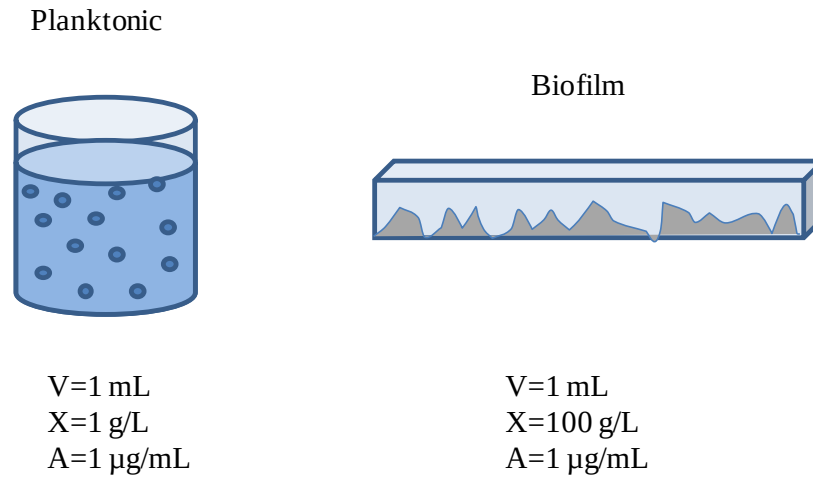


Figure 1. An example case of antibiotic treatment. V = sample volume, X = dry biomass density, V_{biofilm} = biofilm volume (thickness $\times$ surface area), A = antibiotic concentration.

We can treat the planktonic cells with antibiotic (Figure 1), when the sample volume is 1 mL, cell density is 1 g/L, and the antibiotic concentration is 1 $\mu\text{g/mL}$. The amount of antibiotic per gram of planktonic cell is $1 \times 10^3 \text{ } \mu\text{g antibiotic/gram biomass}$ ($1 \text{ } (\mu\text{g/mL}) / (1 \text{ g biomass/L})$). For biofilm systems, we assume that the thickness of biofilm is 100 μm and the biofilm density is

100 g/L. In this case, the amount of antibiotic per gram of cell is 1×10^1 μg antibiotic/gram biomass (1 ($\mu\text{g/mL}$)/(100 g biomass/L)). Simply, the amount of antibiotic per cell mass for the biofilm system is 2 orders of magnitude less than for the planktonic system. If we want to have the same efficiency for biofilm and planktonic systems we will need to add 100 times more antibiotic to the biofilm system. The goal of this research is to test our hypothesis using planktonic cells and biofilms.

For our goal, we used *P. aeruginosa* PAO1 due to its prevalence and the presence of extensive research on these bacteria. We selected tobramycin as the model antibiotic. All cells were grown in a chemostat and planktonic tests were performed using the cells at different densities. The cell densities were adjusted to a desired value by centrifuging and increasing their density. The biofilms were grown in a hollow fiber biofilm reactor. The hollow fiber biofilm reactor allowed us to extend our hypothesis to test biofilm cells by exposing the antibiotics at different concentrations simply by eliminating mass transfer limitations. The planktonic and biofilm samples were tested against different tobramycin concentrations.

2. Materials and methods

2.1. Preparation of inoculum

1 mL frozen stock of *P. aeruginosa* PAO1 was inoculated into 50 mL filled with Tryptic Soy Broth (TSB). The pH was 7.0 ± 0.2 . The flasks were placed on a shaker, set at 150 rpm, ambient temperature and pressure. The typical cell concentration, when plated on R2A agar and grown

for 24 hours in the incubator at 30°C, was 9.4×10^6 CFU/mL. The cells were grown for 16 hours before inoculating in the chemostat.

2.2. Growing *P. aeruginosa* PAO1 in a chemostat

In order to have a continuous source of cells at a constant growth rate, we used a New Brunswick (BioFlo 2000) chemostat with a volume of 2 L. The chemostat was equipped with pH, agitation, temperature, and dissolved oxygen controllers. The sensitivities of the control units for dissolved oxygen, pH, and agitation rate were 0.05 %, 0.1 unit, and ± 1 rpm respectively. Prior to use, the chemostat was autoclaved for 30 minutes at 121°C. The pH was controlled using solutions of 0.1 N NaOH and 0.1 N H₂SO₄. The agitation rate was 350 rpm to maintain a homogeneous culture. The air flow rate was 5 L/h. The chemostat was inoculated with 5 % of an overnight culture and operated in batch mode for 24 hours before switching to continuous feed. The dilution rate was set to 0.0625 h^{-1} .

We followed the procedures described by Beyenal et al., (2001) to grow the microorganism. We used a defined medium with the following composition: Na₂HPO₄, 1830 mg/L (J.T. Baker®); KH₂PO₄, 1350 mg/L (J.T. Baker ®); MgSO₄·7H₂O, 10 mg/L (J.T. Baker®); yeast extract, 10 mg/L (Bacto®); glucose, 1000 mg/L (Fisher®); (NH₄)₂SO₄, and 100 mg/L (J.T. Baker®). The medium was autoclaved at 121°C for 15 min per liter of solution. The ammonium sulfate and yeast extract were autoclaved separately and then added to the growth medium. The glucose was filter sterilized. After the medium was allowed to cool to room temperature, a trace elements solution (1mL/L) was filtered and added to it. The trace elements solution was prepared by

adding $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 357 mg/L; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 228 mg/L; $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 317 mg/L; $(\text{NH}_4)\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$, 231 mg/L; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 127 mg/L; ZnCl_2 , 363mg/L; FeCl_3 , 2160 mg/L; and CaCl_2 , 3700 mg/L.

The microorganism concentration in chemostat was determined using a standard dry-weight method. The glucose concentration was measured using Sigma[®] procedure 510 (Sigma[®] Diagnostics, ST.Louis, MO). Dissolved oxygen and pH were monitored by Ingold[®] dissolved oxygen and pH electrodes were integrated into the chemostat. Dissolved oxygen and glucose concentrations measurements were used to verify steady state conditions. The dissolved oxygen electrode was calibrated in the growth medium while purging the solution with pure nitrogen and air. The electrode that measured pH was calibrated using pH buffers at pH 4 and pH 10.

2.3. Antibiotic treatment of the planktonic cells

After the chemostat culture reached steady state, we used biomass from the reactor for antibiotic tests. The existence of steady state was assumed by having constant dissolved oxygen concentration and biomass density. The chemostat at steady state provided cells at constant density. However, when needed, we adjusted the density of the cells simply by using centrifugation. For example, if we needed to increase concentration of the cells we took, 500 mL culture (50 mL in ten centrifuge tubes) and centrifuge them. Then, we remove the supernatant, and put all the centrifuged cells to 50 mL total volume. We should note that when a volume was taken from the reactor, steady state would need to be reached again before the next experiment. The final concentrated cells were placed in a 50 mL centrifuge tube, then sonicated for 30 s and

centrifuged at 4°C, 6,000 rpm, for 20 min. The supernatant was poured off and cells were re-suspended in phosphate buffer which was identical to the growth medium. Then the samples were treated with 10, 100, or 1000 mg/L of tobramycin for 6 h at room temperature. After antibiotic treatment, the samples were washed with phosphate buffer three times. The samples were diluted and plated following the procedure given by Lewandowski and Beyenal (2007) (Lewandowski and Beyenal 2007). Briefly, serial dilutions were made and inoculated by spread plating to Tryptic Soy Agar (TSA). Plates were incubated at 37°C for 24 h. Number of cells were estimated as Colony Forming Units per ml. Before plating, the cells were washed with phosphate buffer three times (centrifuging 5, 100 rpm for 15 min each). The results are presented as log reduction (logarithm of the ratio of CFUs before and after tobramycin challenge). All the experiments were run in triplicate at different times.

2.4. Hollow fiber biofilm reactor

The experimental setup is shown in Figure 2. A custom-made hollow fiber biofilm reactor (HFBR) was used to grow the biofilms on the hollow fibers. Hollow fibers (5 cm length, and 0.3 cm diameter) were connected to silicon tubing (size 16) by bonding with 5 min epoxy. After drying, it was covered with silicon and left for air drying. The hollow fibers were sterilized with 70% ethanol and rinsed with sterilized phosphate buffer for 15 min each. The rest of the system was sterilized by autoclaving at 121°C for 15 min. HFBRs were inoculated aseptically with 1 ml of inoculum to 40 mL media, using the same medium described for the chemostat experiments. A three-channel peristaltic pump (Masterflex[®] L/S[®] Cole-Parmer Instrument Company) was used at a flow rate of 10 ml/min for the flow conditions. Three identical biofilms were formed for two days at room temperature. They were used for control, recycled, and non-recycled

(static) biofilms. The thickness of the biofilms were measured using a stereo microscope and were statistically similar for each experiment.

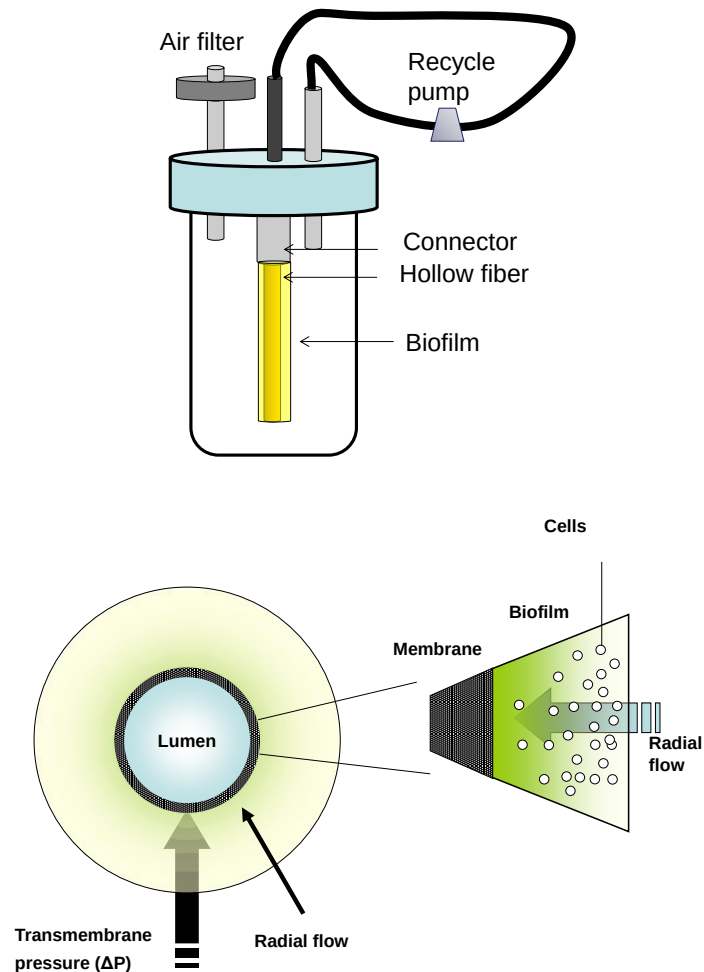


Figure 2. Schematic diagram of hollow fiber biofilm reactor.

2.5. Antibiotic treatment of the biofilms and biofilm-planktonic cells

After 2 days of biofilm formation, two of the hollow fibers were transferred into 40 mL of PBS containing tobramycin. One HFBR was run in with recycle and the other one with no recycle

(static) for 6h. The control biofilm was also treated with the same concentrations of tobramycin after transformed into planktonic cells by dispersion of the biofilm into solution (called biofilm-planktonic). All the antibiotic treatments were repeated for the concentrations of 2.5, 10, 100, and 1000 µg/ml tobramycin.

The control biofilm was detached from the hollow fiber and dispersed into 10 mL of phosphate buffer. Then the biofilm in phosphate buffer was vortexed for 30 s and homogenized for 30 s at 2500 rpm (Lewandowski and Beyenal 2007). Then antibiotic efficiencies evaluated using the same procedures given for the chemostat culture.

3. Results and discussion

3.1. Antibiotic resistance of planktonic culture

By operating a chemostat at steady state, we always produced cells at the same physiological state and specific growth rate. This way, the antibiotic tests were applied to identical cells. It is well known that cells at different growth phases can show different resistance against antibiotics (Brown, Collier et al. 1990) . By using a chemostat we simply avoided this problem.

Figure 3 shows that when the cell density increased, the log reduction decreased. This verified that when the same amount of the tobramycin was used against an increasingly larger bacterial population (higher cell density), the log reduction of cells decreased. The data shown in Figure 3 verifies that the ratio between the amounts of antibiotic to cell density controls the efficiency of the antibiotics. We should note that at low cell densities the log reduction was low and increased

with increasing cell density. This was simply an artifact of the low density of cells. Since initial cell numbers were low, they all were killed due to antibiotic application and log reduction was low. This increased with the increased cell density until a maximum was reached. After the maximum (such as ~ 4 g/L for 1000 $\mu\text{g/mL}$ tobramycin), the efficiency of antibiotic decreased because not all of the cells were killed.

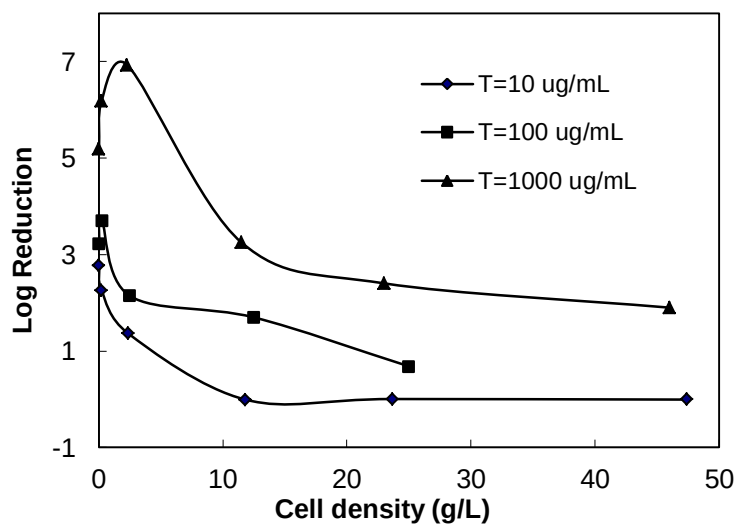


Figure 3. Cell density of planktonic cells versus log reduction when the samples are treated using various concentrations of tobramycin.

Figure 4 shows the variation of log reduction with the ratio between the amounts of tobramycin to total CFU (as $-\log(\mu\text{g T/CFU})$) treated with the antibiotic. The smaller the $-\log(\mu\text{g T/CFU})$ is the more the antibiotic present for the identical CFUs. We found a linear range between 4-6 $-\log(\mu\text{g T/CFU})$ for each tested antibiotic concentration. This indicates that within this range the critical parameter is the amount of antibiotic delivered to the cells. For $-\log(\mu\text{g T/CFU})$ higher

than 6, we found that log reduction to be around zero, indicating that antibiotic was not effective under these conditions. For low $-\log(\mu\text{g T/CFU})$, the log reduction values were different for each applied tobramycin concentrations. The highest log reduction was observed for the highest antibiotic concentration. Most likely for the $-\log(\mu\text{g T/CFU})$ smaller than 4, antibiotic concentrations were more influential in cell death.

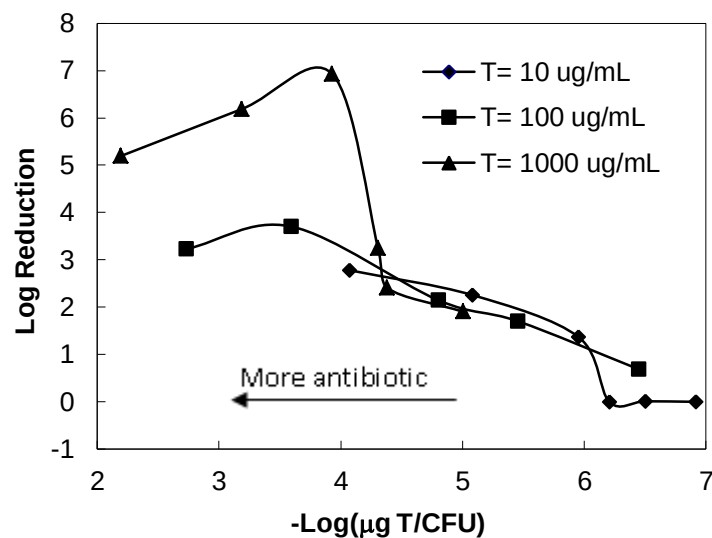


Figure 4. The $-\log(\mu\text{g T/CFU})$ versus log reduction for the planktonic cells. The smaller ($-\log(\mu\text{g T/CFU})$) values corresponds to more antibiotic.

3.2. Antibiotic resistance of biofilms

For hollow fiber biofilms, we treated our biofilms using three different methods. 1) non-recycled only, 2) recycled, and 3) biofilm-planktonic. Each case described in detail below.

Static (non-recycled): In this case, the biofilms grown on hollow fiber membranes were challenged with tobramycin without additional mixing. Tobramycin was delivered to the biofilm by diffusion only. In this mode of treatment, we expect that some of the tobramycin in the bulk solution was available for the cells in the biofilm. This is traditionally how biofilm samples are challenged against antibiotics.

Recycled: In this case, a radial flow through the hollow fiber was applied (Figure 2). This allowed the tobramycin to be delivered to the biofilm *via* convection. In this mode of treatment, we expect that all of the tobramycin in the bulk solution was available for the cells in the biofilm.

Biofilm-planktonic: In this case, we removed the cells from the hollow fiber and re-suspended them in solution. In this mode of treatment, we expect that all of the tobramycin in the bulk solution was available for the biofilm planktonic cells.

As expected, we found similar results for the planktonic cells – the log reduction decreased with the increased $-\log(\mu\text{g T/CFU})$. The log reduction values were different when the samples were challenged with 100 μg tobramycin/mL. The lowest log reduction (~ 4) was observed when tobramycin was only allowed to diffuse through the biofilm (static mode). When we overcome the diffusion limitation by using recycle, the log reduction increased (~ 5.5). There was a statistically significant difference between the log reductions of recycled and static mode ($P = 0.048$). However, when the biofilm cells were suspended, we observed highest log reduction (~ 7.5). The log reduction difference was statistically significant for the biofilm planktonic and

recycled ($P = 0.010$) as well as the biofilm planktonic and static mode ($P = 0.013$). We did not see any differences in log reduction between the three systems when we challenged our samples with 1000 or 10 or 2.5 $\mu\text{g/mL}$ tobramycin concentrations.

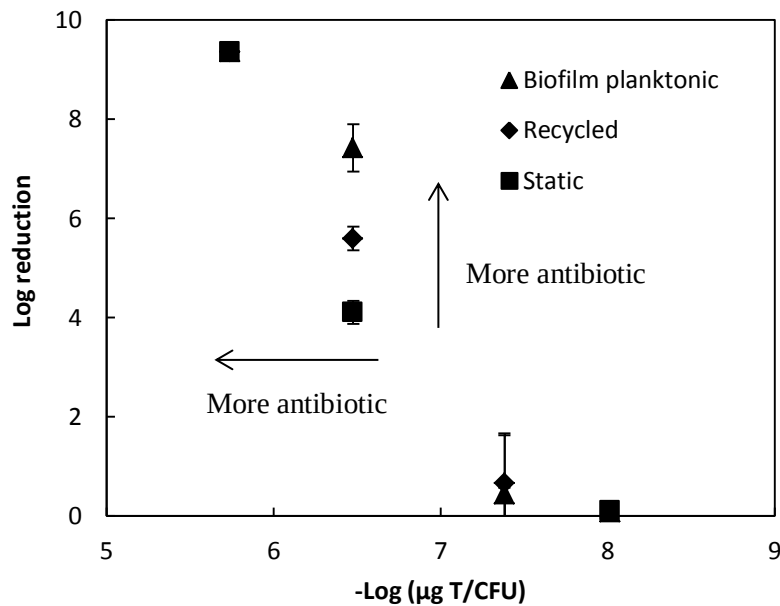


Figure 5. $-\log (\mu\text{g T/CFU})$ versus log reduction for various tobramycin concentrations for different treatments.

Our results suggest that the antibiotic mass to total CFU ratio might be an important parameter to determine the effectiveness of an antibiotic against planktonic cells and biofilms, and that it will be possible to obtain an effective treatment with the ratio lower than 4 log assuming this is not toxic to the patient. We also found a linear trend between 4-6 log ($\mu\text{g T/CFU}$) and this linear trend is consistent with a dose-response relationship. It is well known that there is a relationship

between antibiotic concentration and antimicrobial efficacy and that antibiotics are ineffective in low concentration. It is also known that antibiotics exhibit reduced bactericidal activities at high drug concentrations. However, our research extends past this and demonstrates a range where the concentrations do not matter and the effectiveness controlled by the amount of antibiotic per unit CFU.

Our results also suggest that it will be possible to obtain an effective treatment with the antibiotic mass to CFU ratio lower than 6.5 against *P. aeruginosa* PAO1 biofilms. But the ratio is not a valid parameter in high-dose (1000 µg tobramycin/mL) and low-dose (10 and 2.5 µg tobramycin/mL) tobramycin to determine the effectiveness of the tobramycin against biofilms. It is reported that peak concentration of tobramycin (TOBI=inhalated formulation of tobramycin) measured in sputum samples of CF patients was approximately 1000 mg/L (Geller, Pitlick et al. 2002). It was also shown that tobramycin at this concentration alone has no cytotoxicity (Moreau-Marquis, O'Toole et al. 2009). Therefore, we used this concentration as high-dose concentration of tobramycin in our study and we found that this concentration was highly effective in killing *P. aeruginosa* PAO1 biofilms and planktonic cells at the tested cell densities. Similar to ours, Yu Q et al. (Qianru Yu, Edward F. Griffin et al. 2012) showed that a significant reduction (>4 log) in *P. aeruginosa* PAO1 biofilm when treated with tobramycin in static co-culture model.

Tobramycin is a member of the polycationic aminoglycoside family of antibiotics and has ability to inhibit bacterial protein synthesis at the ribosome (Vicens and Westhof 2002). There are two important steps in uptake of tobramycin into the cell. The first step is to bind its cationic agent to

anionic components in the cell membrane. In the second step, the cationic agent crosses the cytoplasmic membrane and interacts with the ribosomes after destabilizing the outer membrane. The processes in the last step in an energy-dependent process and are most likely dependent on membrane potential (Fraimow, Greenman et al. 1991; Leviton, Fraimow et al. 1995). It has been shown that oxygen availability is an important factor in the tolerance of *P. aeruginosa* biofilms, when applying antibiotics that include tobramycin (Walters, Roe et al. 2003; Borriello, Werner et al. 2004; Field, White et al. 2005). For our planktonic experiments, the solution was saturated with oxygen and we did not expect that oxygen concentration would play a role. However, for biofilm experiments, when we introduced convective flow, we assumed that oxygen limitations would be minimized and tobramycin delivery maximized. Therefore, we expected kill rate to be higher compared to the static biofilm tests. However, when we removed the biofilms and converted them to biofilm planktonic cells, we found that the killing rate was the highest. One of the reasons for the increased kill rate could be increased oxygen availability as well as increased tobramycin availability.

It has been claimed that one of the critical differences between biofilm and planktonic cells is the diffusion of antibiotics (Pereira, Kuehn et al. 2002; Stewart 2003; Eberl and Sudarsan 2008). However, our hollow fiber biofilm reactor allowed us to eliminate diffusion, and contrary to our expectation, we found that the kill rates were different. Recent studies focusing on improving convection through biofilms found that convection improved efficiency of antibiotics (Eberl and Sudarsan 2008; Stewart 2012). However, our results demonstrated that convection could be one of the factors in addition to the ratio of the antibiotics to CFU.

Our unique hollow fiber biofilm reactor allowed us to test biofilm samples in three distinct modes. The first one was the static mode where antibiotics diffuse naturally through the biofilm. The second one had antibiotic transferred through the biofilm using convection. This mode allowed us to have antibiotic delivered bottom part of the biofilm as well as controlling tobramycin mass to CFU ratio by changing tobramycin concentration. The last mode was to biofilm-planktonic. In this mode, we moved biofilm cells to a planktonic phase through dispersion of the biofilm, and tested antibiotic efficiency. There are many attempts to increase tobramycin efficiency against *P. aeruginosa* biofilms such as different antibiotic combinations with tobramycin (Tre-Hardy, Vanderbist et al. 2008; Herrmann, Yang et al. 2010), FDA-approved iron chelators (Moreau-Marquis, O'Toole et al. 2009), natural products including asiatic acid and corosolic acid shown that possess anti-biofilm activity (Garo, Eldridge et al. 2007), and electrical current (Costerton, Ellis et al. 1994; Stewart, Wattanakaroon et al. 1999; del Pozo, Rouse et al. 2009). Our results demonstrated that the ratio of antibiotic to total CFU of biofilms should be considered when the biofilms are challenged with antibiotics.

4. Conclusions

We found that when *P. aeruginosa* PAO1 challenged with tobramycin, there are three distinct zones: 1) low tobramycin where there is no significant log reduction, 2) linear effect zone where the log reduction increase linearly with the logarithm of the amount of tobramycin to total CFU in the sample, and 3) concentration zone where tobramycin concentration controls the log reduction.

When biofilm samples were challenged with tobramycin using different delivery strategies we found that the response depended on the concentrations and strategies. For low and high concentrations of tobramycin we did not see any differences between tobramycin delivered using different mechanisms. However, for a tobramycin concentration of 100 µg/mL we observed three distinct responses indicating the differences in tobramycin delivery.

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CHAPTER 5

CONCLUDING REMARKS & FUTURE WORK

The main conclusion of studying bacteria-substratum interactions is that the biofilm control is dependent on the number of the cells located in the biofilms. It is very relevant starting from the initial number of cells thorough the development into mature biofilm. To control biofilm formation on the surfaces we should know the mechanisms of it. For that reason we first studied the interaction of the cells and substratum under no electron acceptor conditions. We developed a well controlled electrochemical cell and monitor the cells *in situ*. This allowed us to see the importance of the initial number of cells introduced to the system and how it affects the cell respiration.

After the work with cell respiration-surface interaction, we found that by changing the potential of the surfaces biofilm formation could be controlled. We modified the electrochemical cell developed in the first part of this study and used for the electrochemical biofilm control on 316L stainless steel surfaces. We found that the electrochemical reactions taking place on the surface such as oxygen reduction affected the cells to grow into mature biofilm. However, we noticed that the oxygen is required for that kind of system to be able to have continuous electrochemically induced hydrogen peroxide production on the surface.

The work with hydrogen peroxide production raised the question of the effectiveness of the antimicrobials. The concentration of hydrogen peroxide produced in our system was sufficient to inhibit biofilm formation; however a well developed biofilm is at least 10-1000 times more

resistant to be eradicated by the antimicrobials such as biocides or antibiotics. This means we always need to use at least 10-1000 times more antimicrobial to eradicate the biofilms (Mah and O'Toole, 2001). Considering this, we focused on the antibiotic and cell ratio to test our hypothesis.

Overall, we found that there was always a proportional relationship with the cell density and the amount of cells either for planktonic or biofilm cells for a certain amount of antibiotic per cell density. Therefore, findings of this study reveal one of the most important parameters in biofilm a system, which is the number of cells either at the beginning of the biofilm formation or in a mature biofilm.

1. Cells respiration on the various electrode surfaces

Cell respiration on the surfaces is a key point to clarify the bacteria surface interactions in biofilm systems. Our current study showed that the initial number of cells is relevant for the biofilm communities in terms of cell respiration. However, the type of substratum could be effective for the systems where there is no other electron acceptor. The ability of bacteria to respire on the solid surface could give us the insights of biofilm communities. For this work, we determined the cell respiration on glassy carbon surfaces under no electron acceptor conditions. We found that the cells used the surface to transfer their electrons and decreased the potential of the surface. For the future work other substratums could be used. For example, indium tin oxide (ITO) electrodes could be an option. Also this would improve the system in terms of imaging. Since ITO is glass the cells on the electrodes would be easier to monitor. However the resistance of the ITO electrode is usually higher than the glassy carbon electrodes. This could result higher

resistance in the system therefore lower current. Then the potentiostat would have to be modified to be able to monitor the smaller current changes.

2. Electrochemical biofilm control for longer terms

In this study we elucidated the mechanism of action for electrochemical biofilm control.

However, our study was limited to 40 hours. This time interval was sufficient because we aimed to keep it until we saw the effect of the polarization comparing to the control surface. One question could be that how does polarization effect the biofilm formation for longer terms, e.g. 1 month. The hydrogen peroxide concentration produced in our system was able to prevent the attached cells to grow into mature biofilm forty hours. For longer terms it is expected that the same system would have the same impact. However pitting corrosion could be another challenge for the longer term polarization. Even though the concentration of hydrogen peroxide is very low, longer term of polarization of 316L stainless steel might corrode the surface.

For our study we used defined medium and *P. aeruginosa* PAO1 to study the mechanism of the electrochemical biofilm control. For industrial applications, real food material could be used, e.g. raw milk. In that case however, it would be more challenging because of the components of the food material. Though challenging, we suggest that the components of the food, such as total proteins, would not be changed because hydrogen peroxide production on the surface is very low.

3. Density effect in various biofilms having more or less cell density

In this study biofilms we challenged with varying amount of concentrations of tobramycin. This study could be extended for the different biofilms having various biofilm ages. This would support the obtained results. However it is suggested that the trend would be similar considering our hypothesis. Because when the age of the biofilm changes the cell density would be increased.

The same biofilms could also be challenged with 250 mg/L and 500 mg/L tobramycin. We suggest that we could have a linear zone with a linear equation. We could have a standard curve for this interaction. This could be a very relevant tool for the antibiotic applications for biofilms. Because the amount of antibiotic required for biofilms is usually more than the planktonic cells we could have a chart to choose the working antibiotic amount for the desired log reduction. The slope of the linear zone would be constant and the given equation could be used to predict the empirical log reduction for a given antibiotic concentration at certain biofilms with high cell densities.

Appendix A

Supporting information for Chapter 2

MATERIALS

REAGENTS

Hydrochloric acid (HCl)

Sodium Hydroxide (NaOH)

Pipes buffer ($\text{C}_8\text{H}_{18}\text{N}_2\text{O}_6\text{S}_2$)

Sodium Hydroxide (NaOH)

Ammonium Chloride (NH_4Cl)

Potassium Chloride (KCl)

Sodium Phosphate monobasic ($\text{NaH}_2\text{PO}_4\text{H}_2\text{O}$)

Sodium Chloride (NaCl)

Sodium lactate (60 % (w/w))

Nitrilotriacetic acid ($\text{C}_6\text{H}_9\text{NO}_6$)

Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)

Magnesium sulfate monohydrate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$)

Ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)

Calcium chloride dehydrate ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$)

Cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$)

Zinc chloride (ZnCl_2)

Cupric sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)

Aluminum potassium disulfate dodecahydrate ($\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$)

Boric acid (H_3BO_3)

Sodium molybdate dehydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$)

Nickel chloride ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$)

Sodium tungstate

Biotin (d-biotin, $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

Folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$)

Pyridoxine HCl ($\text{C}_8\text{H}_{12}\text{ClNO}_3$)

Riboflavin ($\text{C}_{12}\text{H}_{20}\text{N}_4\text{O}_6$)

Thiamine HCl monohydrate ($\text{C}_{12}\text{H}_{18}\text{N}_4\text{OSC}_2\text{H}_5 \cdot \text{H}_2\text{O}$)

Nicotinic acid ($\text{C}_6\text{H}_5\text{NO}_2$)

d-pantothenic acid hemicalcium salt ($\text{C}_9\text{H}_{17}\text{NO}_5 \cdot 1/2\text{Ca}$)

B12 ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

p-aminobenzoic acid ($\text{C}_7\text{H}_7\text{NO}_2$)

Thioctic acid ($\text{C}_8\text{H}_{14}\text{O}_2\text{S}_2$)

L-glutonic acid ($\text{C}_5\text{H}_9\text{NO}_4$)

L-arginine ($\text{C}_6\text{H}_{14}\text{N}_4\text{O}_2$)

DL-serine ($\text{C}_3\text{H}_7\text{NO}_3$)

70 % Ethanol

Distilled water

Nitrogen (N_2)

EQUIPMENT

Flat-plate flow cell components

- **Stainless steel cover:** Two stainless steel cover (7 x9 cm), one of them is for the top and the other one is for the bottom of the flow cell.
- **Rubber cover:** Five rubber covers (7 x9 cm).
- **Cover glass:** Two microscope cover glass (Gold seal cover glass, 48 x 60 mm, No. 1), one of them is for the top and the other one is for the bottom of the flow cell.
- **Electrode holder:** One polycarbonate holder (7 x 9 cm) to insert electrodes in the flow cell by attaching the electrodes on this holder.
- **Cation exchange membrane:** One cation exchange membrane (Membranes International Inc., CMI-7000, 6 x 8 cm).
- **Polycarbonate block:** Counter electrode and reference electrode will be placed to opening of this block (7 x9 cm).
- **Screws and nuts:** Ten stainless steel screws and ten nuts.

Electrodes

- **Working electrode:** Two glassy carbon electrodes (1.18 x1.18 cm) will be used.
- **Reference electrode:** Ag/AgCl reference electrode will be used.
- **Counter electrode:** One graphite electrode (1 x 2 cm) will be used.
- **Platinum wire:** Two platinum wires (length: 10 cm diameter: 0.04 mm) will be used to attach to the working and counter electrodes.

- **Conductive epoxy:** Chemtronics conductive epoxy will be used to glue the platinum wires to the electrodes.
- **Silicon:** Will be used to cover the glued parts.

Flat-plate flow cell assembly components

- **Glass flow breaks:** Two glass flow breaks will be used to prevent back contamination.
- **Tubing:** 14 size autoclavable Norprene black tubing.
- **Carboys:** Three autoclavable Nalgene carboys with caps each one having two ports.
- **Bacterial air vents:** Autoclavable, PTFE-Membrane 0.2 μm pore size will be spliced into the tubing on waste, nutrient and electrolyte carboy.
- **Peristaltic pump and pump heads:** One pump (Masterflex[®] L/S[®] Digital economy drive, Cole-Parmer Instrument Company) with two heads (Easy-load Masterflex[®] L/S[®], model 7518-60, compatible with 14 size tubing).
- **Clamp stand and clamps:** Will be used to hold the potentiostat alligator clips connected to electrodes in the flow cell.
- **Alligator clips:** Three alligator clips will be used to connect the working, reference and counter electrodes to the potentiostat.

Inoculum preparation

- **Shaker:** Will be used at 150 rpm at room temperature.

- **250 mL Erlenmeyer flask:** Will be used to sterilize the medium which will be used for culture incubation.
- **Conical-bottom sterile disposable centrifuge tube:** This tube will be used for the dilution preparation of the inoculum.
- **Vortex:** Will be capable of mixing the diluted culture enough before inoculation to the flow cell.
- **Pre sterilized syringe:** Sterile syringe that has 10 mL of volume and its needle.
- **Biological safety cabinet:** Will be used to prepare inoculum.
- **Autoclave:** Will be used to sterilize the flat-plate flow cell components, medium, and carboys.
- **Spectrophotometer:** Will be used to measure the optical density (at 600 nm) of the overnight culture and prepared diluted culture before inoculation to the flow cell.
- **Spectrophotometer cuvettes:** Quartz cuvettes which can be used in visible spectrum.
- **Micropipette:** 100-1000 μ l fill capacity.
- **Micropipette tips:** 1000 μ l pipet tips (Fisherbrand) will be sterilized prior to use.

Potentiostat: Gamry; with the software Gamry Instruments Framework™ (Version 5.5, 2008).

Multimeter: Fluke 189 True RMS Multimeter to measure the OCP and resistance of the electrodes prepared.

Ultrasonic bath: Will be used for cleaning of the glassy carbon electrodes.

Inverted fluorescence microscope: Nikon Eclipse Ti-S inverted microscope mounted Nikon DS-Qi1Mc camera. The objective 40x ELWD (Extra-long working distance) will be used. Other brand microscopes which are fluorescence can also be used.

REAGENT SETUP

Inoculum culture media Prepare 100 ml of M1 Minimal media (See below).

M1 Minimal Medium: Prepare 3 L of M1 Minimal media with distilled water in a carboy. Add Pipes buffer ($C_8H_{18}N_2O_6S_2$) 0.91 g/l, Sodium Hydroxide (NaOH) 0.3 g/l, Ammonium Chloride (NH_4Cl) 1.5 g/l, Potassium Chloride (KCl) 0.1 g/l, Sodium Phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) 0.6 g/l, Sodium Chloride (NaCl) 1.75 g/l, mineral solution 10 ml/l. Set pH to 7.0 by using HCl or NaOH. Splice the PTFE-Membrane to one of the ports of the carboy lid. Clamp both ports of the carboy lids loosely close the lid prior autoclaving and autoclave the carboy at 121°C for 20 min. After autoclaving, tighten the lid of the carboy; unclamp the tubing of the carboy which has the PTFE-Membrane. Allow enough time to cool down. Add vitamin solution 10 ml/l, amino acid solution 10 ml/l, sodium lactate (60 % (w/w)) 1.1206 g/l distilled water in the biosafety hood. Connect the N_2 to the inlet of the feed carboy inlet through the filter and remove the oxygen in the medium by pumping Nitrogen 4-5 h prior to use the medium. Depending on the media volume the cooling time will increase. Allow at least 12h to cool down. To speed up the cooling process the carboy can be put under running cold water.

Mineral Solution: Add Nitrilotriacetic acid ($C_6H_9NO_6$) 1.5 g/l, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) 3 g/l, magnesium sulfate monohydrate ($MnSO_4 \cdot H_2O$) 0.5

g/l, ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) 0.1 g/l, Calcium chloride dehydrate ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$) 0.1 g/l, cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) 0.1 g/l, zinc chloride (ZnCl_2) 0.13 g/l, cupric sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) 0.01 g/l, aluminum potassium disulfate dodecahydrate ($\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$) 0.01 g/l, boric acid (H_3BO_3) 0.01 g/l, sodium molybdate dehydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) 0.025 g/l, nickel chloride ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$) 0.024 g/l, sodium tungstate 0.025 g/l distilled water. Set pH to 7.0 by using HCl or NaOH.

Vitamin solution: Biotin (d-biotin, $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) 0.002 g/l, folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$) 0.002 g/l, pyridoxine HCl ($\text{C}_8\text{H}_{12}\text{ClNO}_3$) 0.01 g/l, riboflavin ($\text{C}_{12}\text{H}_{20}\text{N}_4\text{O}_6$) 0.005 g/l, thiamine HCl monohydrate ($\text{C}_{12}\text{H}_{18}\text{N}_4\text{O}_5\text{SCl}_2 \cdot \text{H}_2\text{O}$) 0.005 g/l, nicotinic acid ($\text{C}_6\text{H}_5\text{NO}_2$) 0.005 g/l, d-pantothenic acid hemicalcium salt ($\text{C}_9\text{H}_{17}\text{NO}_5 \cdot 1/2\text{Ca}$) 0.005 g/l, B12 ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) 0.0001 g/l, p-aminobenzoic acid ($\text{C}_7\text{H}_7\text{NO}_2$) 0.005 g/l, thiocctic acid ($\text{C}_8\text{H}_{14}\text{O}_2\text{S}_2$) 0.005 g/l distilled water. Set pH to 7.0 by using HCl or NaOH. Filter-sterilize and keep it at refrigerator.

Amino acid solution: L-glutonic acid ($\text{C}_5\text{H}_9\text{NO}_4$) 2 g/l, L-arginine ($\text{C}_6\text{H}_{14}\text{N}_4\text{O}_2$) 2g/l, DL-serine ($\text{C}_3\text{H}_7\text{NO}_3$) 2 g/l distilled water. Set pH to 7.0 by using HCl or NaOH. Filter-sterilize and keep it at refrigerator.

70% ethanol Add 368 ml of 95% ethanol to 132 ml of sterile distilled water. Can be prepared and stored at room temperature for up to 2 weeks.

1 M HCl solution Add 8.2 ml of 37.5% HCl to 100 ml distilled water.

Electrolyte solution Prepare 3L of 3M NaCl solution by adding 175.32 g/l distilled water. Autoclave as stated in ‘Continuous flow media’ part.

Bacterial culture and inoculum preparation

- 1 ml frozen *Shewanella oneidensis* MR1 stock sample in a vial will be used from the stock culture collection (stored at -70°C). Thaw the frozen stock culture, inoculate to sterilized 100 ml M1 Minimal medium and incubate on a shaker at 150 rpm at room temperature (approximately 25°C) for 24h.
- Transfer 1 ml of the grown culture into a quartz cuvette by using a micropipette in order to measure the optical density. Cover the top of the cuvette with cuvette cap or parafilm and measure the optical density at 600 nm.
- Dilute the grown culture accordingly in a 50 ml pre sterilized test tube with sterilized M1 minimal media. The optical density of the inoculum should be around 0.005.
- Transfer 10 ml of inoculum to the pre sterilized syringe.

All microorganisms should be handled according to the biosafety recommendations specific for each individual species. Contaminated media and all equipment should be decontaminated before disposal or reuse.

Estimated time for reagent setup is 4 h (plus 24 h incubation time).

EQUIPMENT SETUP

Prepare the electrodes

- 1) Mix equal amounts of Part A and Part B in a clean weighting dish. Mix thoroughly for at least 2 minutes.
- 2) Apply sufficient amount of the mixture to the side of each glassy carbon electrodes and graphite electrode.
- 3) Attach the platinum wires within 5 minutes.
- 4) Leave for air drying for at least 5h.
- 5) Cover the epoxy connection with silicon.
- 6) Allow it air dry for 24h.

Epoxy connection and silicon should be completely dry.

Part A and Part B should be mixed in equal amounts; otherwise connection would be poor and take more time to dry.

Measure the resistance of the electrodes

- 1) Use multimeter to measure the resistance of the electrodes.
- 2) First measure the resistance of the dry epoxy mixture.
- 3) Put the black and red probes on the dry mixture, resistance should be less than $0.5\ \Omega$.
- 4) Put black probe on the glassy carbon electrode and the red probe on the platinum wire, resistance should be around $5\ \Omega$.

The resistance should not be too high, if so that means the connection is poor and should be prepared again.

Clean the glassy carbon electrodes

- 1) Put the glassy carbon electrodes in a 100 ml beaker filled up to 20 ml 3 M HCl solution. Cover the top of the beaker with parafilm and leave it for 24h.
- 2) Rinse the glassy carbon electrodes with distilled water.
- 3) Put the glassy carbon electrodes in distilled water in a beaker and sonicate it in an ultrasonic bath for 1 h.
- 4) Leave the glassy carbon electrodes for air drying.

Wear laboratory gloves. Do not touch the glassy carbon electrodes. Hold them with a forcep when necessary.

Prepare Ag/AgCl reference electrode

- 1) Cut a piece of silver wire approximately 7-10 cm.
- 2) Clean the surface of the silver wire with 600-grit sandpaper.
- 3) Dip the wire in 3 M HNO₃, and rinse with distilled water.
- 4) Connect the silver wire to the anode of the power supply, and close the circuit by connecting a graphite rod to the cathode of the power supply.
- 5) Place both the silver wire and the carbon rod into a 0.1 M HCl solution.
- 6) Turn the power supply on, set the voltage between 0.1 and 0.5 V DC, and leave the electrodes, the silver wire, and the carbon rod in the HCl for about 90 min.
- 7) Select the voltage to be used by slowly increasing it until small gas bubbles are observed on the silver wire.
- 8) After chlorination, the electrode should be aged for 1-2 days in dilute HCl (0.1 M).

Applying a low voltage (0.1 V DC) and leaving it overnight produces a smoother AgCl layer; if there is no hurry to complete the electrode, a lower voltage can be applied and waited overnight. To obtain electrodes with a stable potential, high-purity silver with a smooth surface and high-purity HCl should be used.

Preconditioning of cation exchange membrane

- 1) Immerse the cation exchange membrane in 3 M NaCl solution.
- 2) Keep the membrane in the solution for several hours prior to use.

Calibration of potentiostat

- 1) Connect the alligator clips of working electrode, counter electrode and reference electrode ports of the potentiostat to the calibration cell (Gamry Instrument Universal Dummy Cell).
- 2) Open Gamry Framework™.
- 3) Click on Experiment.
- 4) Click on Utilities.
- 5) Click on Calibrate instruments.
- 6) Click on OK.

Prepare flat-plate flow cell

- 1) Place the bottom stainless steel and insert the screws.
- 2) Place the rubber on the stainless steel.

- 3) Place the cover glass which is previously washed with distilled water and air dried, on the rubber.
- 4) Place the polycarbonate block.
- 5) Place the polycarbonate electrode holder in a reverse position.
- 6) Place the rubber on the polycarbonate electrode holder.
- 7) Place the proton exchange membrane on the rubber.
- 8) Place the rubber on the proton exchange membrane.
- 9) Place the polycarbonate block.
- 10) Insert the counter electrode in this polycarbonate block and extent the tip of the platinum wire through the inlet port of the block.
- 11) Insert the reference electrode from the other port of the polycarbonate block.
- 12) Place the cover glass which is previously washed with distilled water and air dried, on the polycarbonate block.
- 13) Place the rubber on the cover glass.
- 14) Place the top stainless steel on the rubber.
- 15) Place the nuts to the screws and tighten.

Start tightening the screws in turn; and little by little. Do not tighten the screws too much, leave them loosely before autoclaving.

Sterilize flat-plate flow cell

- 1) Wrap the flat-plate flow cell with aluminum foil.
- 2) Wrap all the tubing necessary with aluminum foil.
- 3) Autoclave them at 121°C for 15 min.

- 4) After autoclaving, put them to the biosafety cabinet.
- 5) Check the cover glasses and tighten the screws.
- 6) Connect the tubing, flat-plate flow cell, media and waste carboys.

Setup whole system

- 1) Place the flat-plate flow cell with the tubing close to the microscope and potentiostat.
- 2) Place the media tubing to the pump head.
- 3) Place the electrolyte tubing to the pump head.
- 4) Connect working electrode, counter electrode and reference electrode to the alligator clips of the potentiostat.

PROCEDURE

- 1) Connect the tubing of Nitrogen gas tank to the inlet of the feed carboy, and start pumping nitrogen.
- 2) Run pump at the rate of 0.4 ml/min for the feed and salt solution.
- 3) Make sure there is not any bubble in the flow cell.
- 4) Stop pumps.

Nitrogen gas will be used to make the environment anaerobic, therefore there should be enough amount of gas during the experiment.

Place the flow cell system in a tub or tray to contain any leaks that may occur.

Measurements before inoculation • TIMING 1h

- 5) Measure the resistance of the system by using Gamry Framework™.

- 6) Measure Open Circuit Potential (OCP) of working electrodes by using Multimeter.
- 7) Run Cyclic Voltammetry using Gamry Framework™.
- 8) Take the images with 40x objective.

Depending on the scan rate when running Cyclic Voltammetry timing may vary. Slower scan rates will result increased timing.

Polarization

- 9) Start pumps.
- 10) Setup the potentiostat polarization conditions.
- 11) Start polarization of the system by running Repeating Chronoamperometry of Gamry Framework™. Allow for polarization for at least 30 min to get the back ground polarization data.
- 12) Stop feed pump and clamp the inlet of the flow cell.
- 13) Inoculate aseptically 10 ml of inoculum ($OD_{600} = 0.005$) from the inoculation port of the flow cell.
- 14) Wait for 2h for the initial attachment.
- 15) Remove the clamp and start feed pump.
- 16) Allow the washing out of the planktonic cells in the flow cell for 10 minutes.

Measurements during polarization

- 17) Measure OCP of nonpolarized electrode by using Multimeter.
- 18) Repeat measuring OCP every 2h.

19) Place the flow cell on the inverted microscope and take the images with 40x objective.

20) Take at least 20 images, save them in the same folder.

21) Repeat taking images every 2h.

Frequency of taking images can be adjusted depending on the doubling time. Take the images as quick as possible.

Measurements after polarization • TIMING 30 min

22) Run Cyclic Voltammetry Gamry Framework™.

23) Stop polarization.

24) Disconnect the alligator clips of the working electrode, counter electrode and reference electrode.

25) Stop pump and take the flow cell system close to the biosafety cabinet and place the flow cell into it.

26) Clamp the tubing of the feed, electrolyte and waste carboy, disconnect it and wrap the tips with aluminum foil.

27) Loosen the screws of the flow cell and wrap with aluminum foil.

Be sure to wear lab coat and gloves. Apply carefully microbiological precautions.

28) Autoclave the flow cell system for 15 min at 121 °C.

29) Autoclave the carboys for 20 min at 121 °C.

Calculations • TIMING 30 min

- 30) Open the pictures taken for both polarized and nonpolarized electrodes.
- 31) Calculate the cell numbers per unit area.
- 32) Calculate current/cell for each time intervals.

Appendix B

Supporting information for Biofilm Imaging

1. Growing biofilm in a flat-plate flow cell

Media and growth conditions

Pseudomonas aeruginosa-GFP tagged was grown in Carbenicillin (150 µg/ml) supplemented TSB (Tryptic Soy Broth) overnight (24h) on a shaker at 150 rpm at room temperature.

Biofilm formation

Flat-plate flow cell was used for biofilm formation. Feed was pumped at the rate of 0.4 mL/min. Feed was stopped and 10 mL of culture ($OD_{600}=0.007$) was inoculated to the flow cell. After 2h of initial attachment feed was pumped through the system.

Microscopy and structural analysis of biofilms

Images of the cells attached on the surface of the cover glass were monitored by epifluorescence-inverted microscope during biofilm formation through the cover slip placed at the bottom of the flow cell. 40x objective was used to image the single cells.

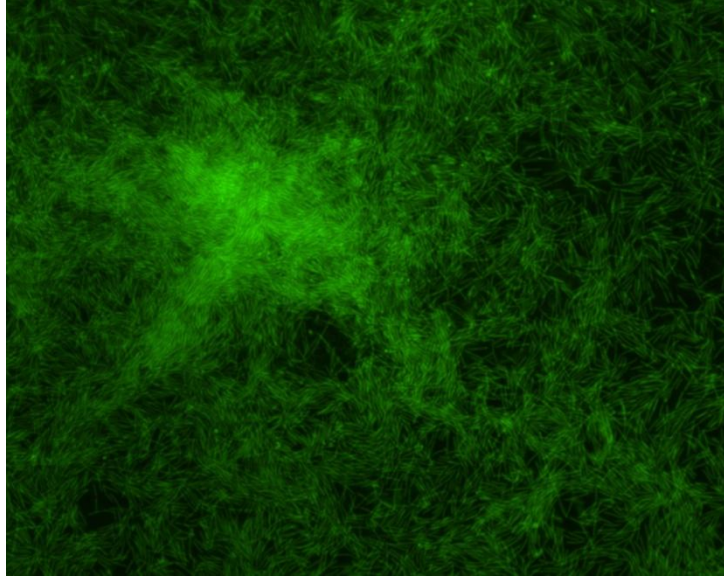


Figure 1. *Pseudomonas aeruginosa* PAO1 biofilm grown on a glass cover slip.

2. Scanning Electron Microscopy (SEM) standard fixation protocol

Geobacter grown on carbon felt electrodes fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1M phosphate buffer overnight followed by rinsing with 0.1M phosphate buffer (3x10 mins each). Samples were dehydrated gradually by using 30, 50, 70, 95% alcohol (10 mins each), and 100% alcohol (3x10 mins each). Hexamethyldisilazane (HMDS) was used for overnight drying. Then the samples imaged with FEI SEM.

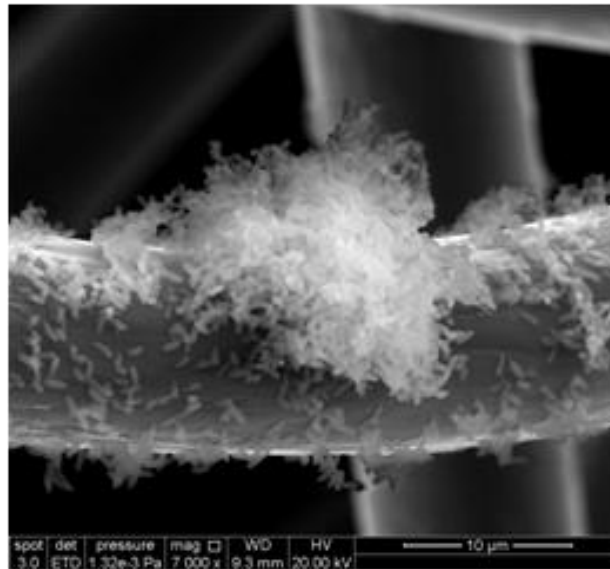


Figure 2. *Geobacter* sp. biofilm grown on carbon felt.

3. Transmission Electron Microscopy (TEM) protocol for biofilms grown on glassy carbon electrode

Glassy carbon electrode was removed from the flat plate reactor and placed in a Petri dish.

Fixation was done with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1M phosphate buffer overnight followed by rinsing with 0.1M phosphate buffer (3x10 mins each). Biofilm is scrapped into eppendorf tube by rinsing with same buffer. The sample was dehydrated gradually by using 30, 50, 70, 95% alcohol (10 mins each), and 100% alcohol (3x10 mins each). Then the sample was rinsed with LR white and ethanol (1:1) (10 min) after that LR white resin was added and left overnight at room temperature. The sample embedded in resin overnight in an oven at 60°C. After the polymerization, the sample can be taken out and stored in a dry place. Using the block this sections were prepared.



Figure 3. Thin section Transmission Electron Microscopy images of *Geobacter* sp.

4. Whole cell TEM protocol for cultures

1ml of *Paenibacillus* sp. cultures in Na-bicarbonate and Pipes buffer were taken into eppendorf tubes at the time intervals of 0, 24, 48, 72h. The samples were centrifuged at 10000rpm for 4 min and supernatant discarded. 0.5ml fixation solution of 2% glutaraldehyde, 2% paraformaldehyde in 0.1M phosphate buffer was added on the pellets, vortexed and kept at 4°C overnight. From fixed samples 5µl aliquots placed onto carbon grids. Each grid was kept for 4 min and rinsed with double-distilled water 3 times. After that the samples were stained with 1% Uranyl acetate for kept in dark for 1.5 min. The samples were rinsed with double-distilled water and left overnight drying in a vacuum dessicator.

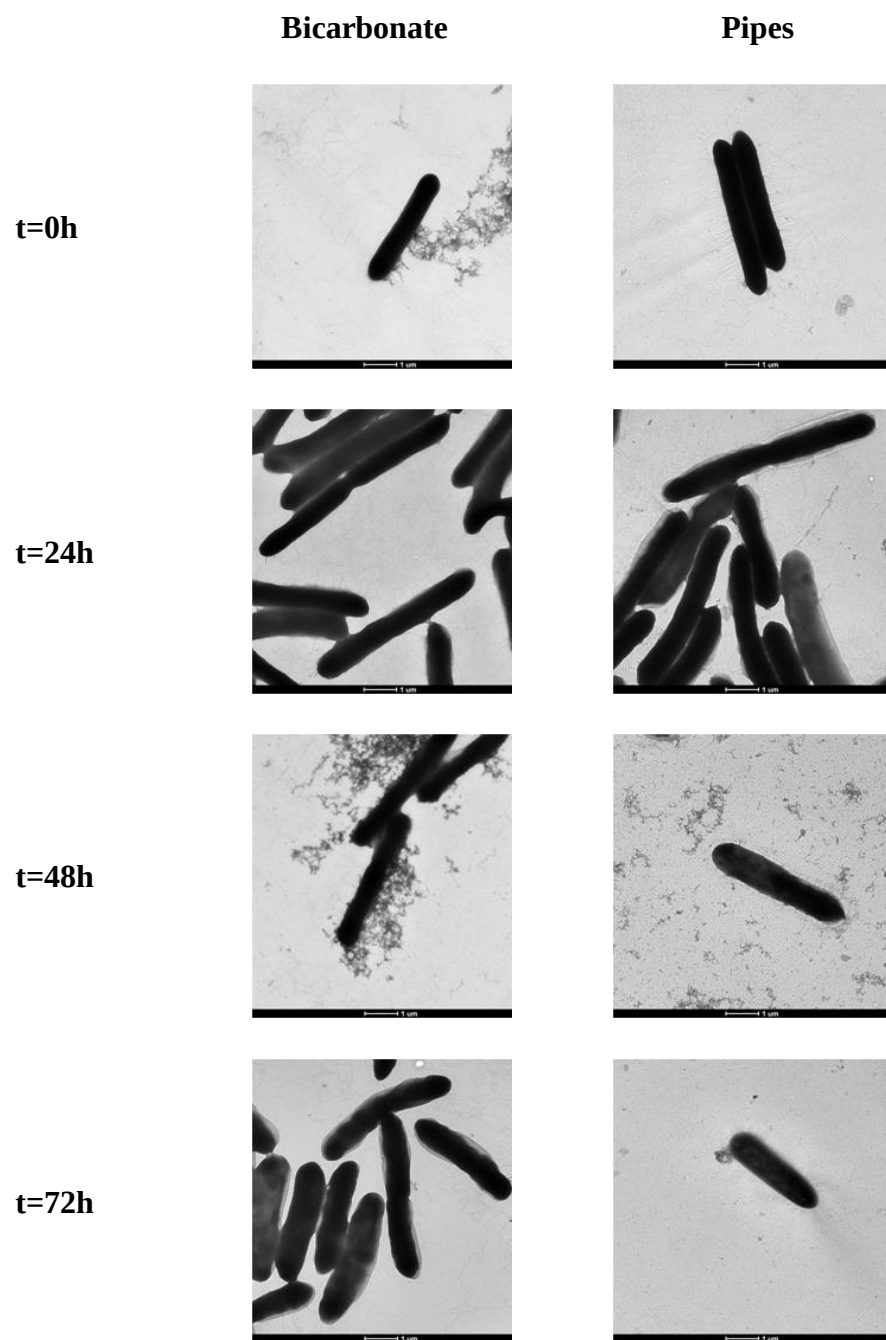


Figure 4. Whole cell TEM images of *Paenibacillus* sp. cultures in Na-bicarbonate and Pipes buffer

5. Live/Dead staining protocol

After 40h of treatment both polarized and non-polarized 316L SS surfaces removed aseptically from the flat plate flow cell and placed in a petri plate. We used a Live/Dead BacLight bacterial viability kit (Molecular Probes Inc., Eugene, OR) to determine the viability of the *P. aeruginosa* (GFP tagged) cells. The Live/Dead BacLight bacterial viability kit contains two separate solid dyes; components A (SYTO 9) and B (propidium iodide). A dye solution was prepared by dissolving the contents of components A and B in separate vials containing 2.5 ml of filter-sterilized deionized water (2X solution). These separate solutions were blended (1:1) and used for biofilm staining. The final concentration of the dye solution was 6 μ M SYTO 9 and 30 μ M propidium iodide. Coupons were stained immediately by adding the stain solution gently to the top of the 316L SS surfaces. The petri plate was then covered with a cover dish and aluminum foil and incubated at room temperature. Then the coupons were gently rinsed with filtered 0.9% NaCl to remove excess stain. The stained coupons were imaged using an inverted microscope (Nikon Eclipse Ti-S, 40x ELWD objective) mounted with Nikon DS-Qi1Mc camera.

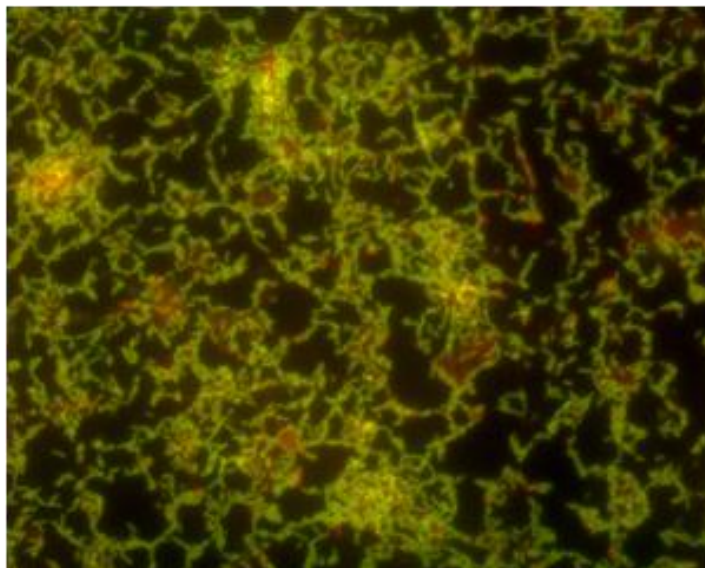


Figure 5. Live/dead image of *Pseudomonas aeruginosa* PAO1. Red cells are dead, green cells are alive cells.

6. Confocal Scanning Laser Microscopy (CSLM) protocol

0.2 ml of *L. monocytogenes* EGDe (tagged with green fluorescent protein-GFP) stock culture added to 10 ml sterile Brain Heart Infusion Broth (BHIB) and incubated at 30 °C at 150 rpm for 12-16 h. 0.3 ml was added to 30 ml of fresh medium. Medium was supplemented with 8 µl/ml erythromycin. Biofilm was grown on stainless steel coupon inserted in a flat plate flow cell and run at room temperature for two weeks.

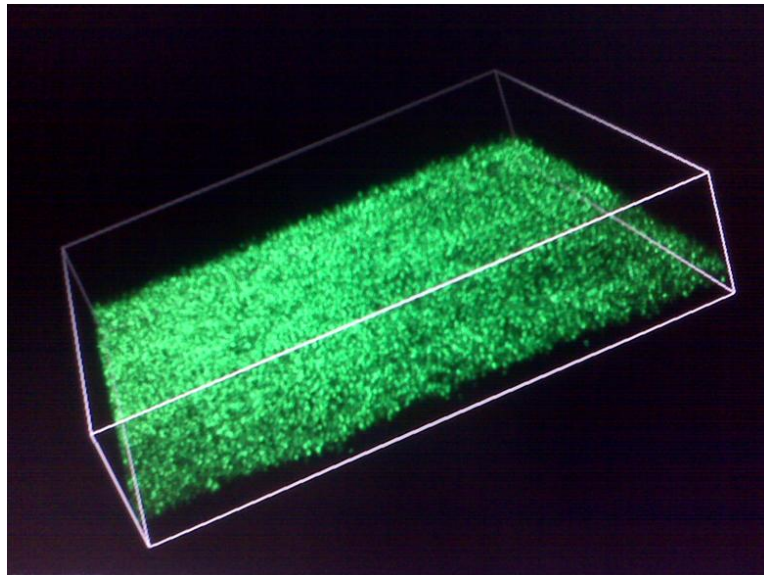


Figure 6. *L. monocytogenes* EGDe biofilm grown on stainless steel surface in a flat-plate flow cell.