Role of biological electron mediators in microbial extracellular electron transfer

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Role of biological electron mediators in microbial extracellular electron transfer

By: Md Mahamudul Hassan

A thesis submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy
In Environmental Engineering

School of Engineering and Information Technology
Murdoch University
WA, Australia

July 2018
Declaration

I hereby declare that this dissertation is my own work and that, to the best of my knowledge, contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

Md Mahamudul Hassan
July 2018
Abstract

Electron mediators are redox active compounds capable of mediating electron transfer from a donor to acceptor. In microbial systems, electron mediators play a key role in extracellular electron transfer processes to assist the bacteria to thrive under unusual environmental conditions. Electron mediators are known to facilitate electron transfer from the bacterial cells to their electron acceptors which are insoluble (e.g. Fe$^{3+}$, Mn$^{4+}$) or toxic (e.g. oxygen for anaerobes). Interspecies electron transfer between different microbial species is also known to be driven by electron mediators. In this case, one species uses the oxidized mediator as electron acceptor and reduces it while the other species uses the reduced mediator as electron donor. The involvement of electron mediators in these electron transfer processes has led to extensive investigation to elucidate their contribution in microbial ecosystems.

The aim of this thesis is to investigate the role of microbially produced electron mediators in facilitating microorganisms to thrive in selected environments that are of human concern. In this study, a novel electrochemical tool was developed that allows characterization of the electron mediators more effectively than the conventional techniques. The proposed method offered much better sensitivity and resolution compared to the conventional technique in detecting electron mediators.

Conventional electrochemical studies use the three-electrode electrochemical cell which is equipped with only one controllable working electrode (WE). The other two electrodes serve as counter and reference electrodes. The traditional one-WE setup is based on the oxidation or reduction of the target molecule at different time interval as for example used in cyclic voltammetry. Having only one WE does not allow mimicking redox condition of the microbial systems where oxidation and reduction occur simultaneously.
In order to test for the presence of redox active mediators, a new apparatus and technique was developed that consists of two independently controllable WEs which enable the generation of redox gradient between the WEs to allow simultaneous oxidation and reduction of the target redox active mediator. By using this redox gradient generating property, a new method was developed that characterizes electron mediators within a thin layer microscale (250 µm) system without the need of a bulk solution and associated mass transfer.

Electrochemical properties of electron mediators were characterized by stepwise shifting a “voltage window” (maintaining 0.05 V potential difference between two WEs) within a range of potentials (between –1 V and +0.5 V vs. Ag/AgCl) and monitoring the establishment of steady equilibrium current in both WEs. The resulting current difference between two WEs was recorded for each voltage step of the “voltage window”. Results indicated that this technique enabled identification (by the distinct peak locations at the potential scale) and quantification (by the peak of current) of individual mediators as well as several mediators in an aqueous mixture. This technique enabled the precise determination of the mid-potential of hexacyanoferrate (HCF), riboflavin (RF) and two mediators from the pyocyanin-producing P. aeruginosa (WACC 91) culture. The capability of Twin-WE approach in detecting unknown electron mediators from a microbial culture confirms its suitability in studying microbial extracellular electron transfer (EET) processes.

The Twin-WE electrochemical cell was used to investigate the role of the bacterial mediator PYO in electron transfer processes accomplished by its producer P. aeruginosa (PA), a high impact bacterium from human health perspective. Pyocyanin (PYO) is a redox active compound present in the biofilm of P. aeruginosa and believed to mediate an electron transfer from PA cells to oxygen for assisting PA to respire under oxygen limited condition. In contrast to widely held belief, this study shows that reduced PYO
(RedPYO) is not readily oxidized by oxygen unless catalyzed by living cells. The results are supportive to a scenario in which PYO can extract electrons from other living cells by oxidizing their NADH. The resulting RedPYO can be utilized as electron donor for oxygen or nitrate respiring PA cells. While this PYO mediated electron transfer resembles syntrophic interspecies electron transfer, it suggests, in this case, the existence of a not yet described form of energy parasitism. The discovery of this parasitic life style puts a new perspective on the role of PYO in biofilms, its natural soil environment and host infections.

The existence of a similar electron extracting mechanism of PYO was also investigated in microbially influenced corrosion (MIC). MIC is a complex bio-electrochemical process where the exposure of the metal to microorganisms and their metabolic products causes dissolution of metal ions. Corrosion of steel occurs due to the existence of simultaneous anodic and cathodic reactions on the steel surface. At the cathodic site, steel loses electrons which consequently causes the dissolution of ferrous ions at the anodic site. Under aerobic condition, steel loses electrons from the cathodic site to oxygen. MIC has been described as bacteria rely on mediators to use electrons from the cathode under anaerobic conditions. The potential role of bacterial mediators to influence corrosion in the presence of oxygen has not been investigated yet.

The capability of PYO to extract electrons from living cells was translated to electron extraction from corroding steel. Results showed that PYO can more efficiently harness electrons from the steel than oxygen alone. The reduced PYO thus generated (RedPYO) subsequently can transfer electrons to oxygen. The corrosion rate as determined by the release of dissolved iron was increased by two-fold when carbon steel was exposed to PYO compared to the exposure to a PYO free electrolyte under oxygen saturated environment. This increase in corrosion rate can be explained by the existence of a PYO mediated electron flow from the steel to the oxygen which accelerated the cathodic half
reaction. PA cells can also benefit from this electron flow to generate cellular energy (ATP) using RedPYO as the electron donor for oxidative phosphorylation. Hence, PA and PYO containing biofilms could be described as catalyst of the cathodic reaction of corroding iron. To our knowledge, this is the first study to demonstrate the role of a biological electron mediator in influencing aerobic corrosion by cathodic stimulation.

Overall, this thesis has contributed towards improving the understanding of microbial mediators, their detection and possible role in microbial consortia and in interaction of microbes with reducing surfaces such as steel constructions.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>II</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>III</td>
</tr>
<tr>
<td>TABLE OF CONTENT</td>
<td>VII</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>VIII</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>X</td>
</tr>
<tr>
<td>PUBLICATIONS AND CONFERENCES</td>
<td>XI</td>
</tr>
<tr>
<td>STATEMENT OF AUTHORS’ CONTRIBUTION</td>
<td>XII</td>
</tr>
<tr>
<td><strong>CHAPTER 1</strong></td>
<td>1</td>
</tr>
<tr>
<td>Introduction and aim</td>
<td></td>
</tr>
<tr>
<td><strong>CHAPTER 2</strong></td>
<td>27</td>
</tr>
<tr>
<td>New method for characterizing electron mediators in microbial systems using a thin-layer twin-working electrode cell</td>
<td></td>
</tr>
<tr>
<td><strong>CHAPTER 3</strong></td>
<td>47</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> uses pyocyanin to extract energy from living cells</td>
<td></td>
</tr>
<tr>
<td><strong>CHAPTER 4</strong></td>
<td>69</td>
</tr>
<tr>
<td>Pyocyanin accelerates <em>Pseudomonas aeruginosa</em> associated aerobic steel corrosion</td>
<td></td>
</tr>
<tr>
<td><strong>CHAPTER 5</strong></td>
<td>96</td>
</tr>
<tr>
<td>Conclusion and outlook</td>
<td></td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>101</td>
</tr>
</tbody>
</table>
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*Hassan*

*Murdoch, Perth*  
*Winter 2018*
To my parents
Publications and conferences

The following outcomes were derived from this thesis:

1. **Peer reviewed publications**

   Hassan, M.M., Cheng, K.Y., Ho, G., Cord-Ruwisch, R. *Pseudomonas aeruginosa* uses pyocyanin to extract energy from living cells. Submitted (Chapter 3)

   Hassan, M.M. and Cord-Ruwisch, R. Pyocyanin accelerates *Pseudomonas aeruginosa* associated aerobic steel corrosion. To be submitted (Chapter 4)

2. **Conference presentation**
   7th Euro Biosensors and Bioelectronics conference
   Berlin, Germany, July 10-11, 2017

3. **Award**
   Best Research Poster in the School of Engineering and IT, Murdoch University, 2017.
   Hassan, M.M., Cheng, K.Y., Ho, G., Cord-Ruwisch, R. New method for characterizing electron mediators in microbial systems using a thin-layer twin-working electrode cell.
Statement of authors’ contribution


<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution</th>
<th>Overall Percentage (%)</th>
<th>Signature</th>
</tr>
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<tbody>
<tr>
<td>Md Mahamudul Hassan</td>
<td>Designed and conducted all experiments, analyzed and processed the data, composed the contents of the manuscript from initial draft to final submission</td>
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<td>Ka Yu Cheng</td>
<td>Advised on experimental setup and coulomb counting experiment, discussed the results and commented on the manuscript.</td>
<td>5</td>
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</tbody>
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2. Hassan, M.M., Cheng, K.Y., Ho, G., Cord-Ruwisch, R. *Pseudomonas aeruginosa* uses pyocyanin to extract energy from living cells. (Chapter 3)

<table>
<thead>
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<th>Contributor</th>
<th>Statement of contribution</th>
<th>Overall Percentage (%)</th>
<th>Signature</th>
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<tbody>
<tr>
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<td></td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution</th>
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<th>Signature</th>
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<td>Advised on experimental design, directed the study, discussed the results and critically reviewed the paper.</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>
1.1. Background

The survival of a respiratory organism depends on the generation of energy (ATP) by operating an electron flow between electron donor and acceptor through their electron transport chain. Microorganisms oxidize organic compounds as their sole carbon and energy (electron donor) source while for accomplishing respiration, a wide range of compounds can be utilized as the terminal electron acceptor such as oxygen (O₂), nitrate (NO₃⁻), sulfate (SO₄²⁻), insoluble iron (Fe³⁺), carbon dioxide (CO₂), sulfur (S⁰) etc. Some bacteria such as *Shewanella* sp. (Pandit et al., 2014), *Geobacter sulfurreducens* (Bond & Lovley, 2003), *Geothrix fermentans* (Bond & Lovley, 2005), *Pseudomonas aeruginosa* (Rabaey et al., 2005) etc. are electroactive in nature and after oxidizing the organics, they can utilize a suitably poised electrode (anode) as their electron acceptor.

The unique property of this group of bacteria in using anode as the electron acceptor plays the key role in microbial fuel cell (MFC) technology to generate electricity from waste (Logan & Regan, 2006; Rabaey & Verstraete, 2005). Electroactive bacteria (electricigens) have also been reported to accept electrons from the electrode (cathodophilic) which enhances the electron transfer reaction from the electrode to oxygen in the cathodic chamber of MFC (Milner et al., 2016). Interestingly, the existence of some unique electricigens have been described which can perform both anodic (electron donation to the electrode) and cathodic (electron receiving from the electrode) reactions (Cheng et al., 2012; Cheng et al., 2010). The driving force for this electron flow from the organics to anode or from the cathode to electron acceptors has been suggested to be dependent on the electrode potential (Dennis et al., 2016).
Chapter 1. Introduction and aims

The electricigens or their redox active metabolites has already been reported to play important roles in different processes linked to human interest such as disease development e.g. Crohn’s disease (Khan et al., 2012), maintaining stable human health e.g. metabolites of human gut biofilms (LeBlanc et al., 2013), destructive bioprocesses e.g. microbially influenced metal corrosion (Beech & Sunner, 2004; Enning & Garrelfs, 2014) and bioprocesses advantageous for human e.g. bioremediation of environmental pollutants (Luo et al., 2009), production of methane, hydrogen, hydrogen peroxide etc. (Logan et al., 2008; Rozendal et al., 2009). The direct involvement of electroactive microorganisms in these processes has made electricigens an interesting subject for further investigation.

1.1.1. Electroactive Biofilm (EABF)

Biofilm is the most common and persistent structure of bacteria which exerts properties different from their planktonic counterparts (Flemming et al., 2016). Biofilms that can establish an electrochemical connection with a conductive surface on which they grow are regarded as electroactive biofilm (Borole et al., 2011; Erable et al., 2009). An electroactive biofilm may also support the growth of other bacteria which cannot directly interact with an electrode, but are able to perform other functions such as breakdown of organics or utilization of other electron donor or acceptors (Borole et al., 2011). EABFs are ubiquitous and can be found in natural environments such as soil, marine sediment or in systems rich in different microbial communities (e.g. sewage sludge, activated sludge etc.) (Erable et al., 2009).

Over the past decade, electroactive biofilms have been a popular subject of research mainly attributed to the strong desire to improve the efficiency of various bioelectrochemical system (BES) technologies such as microbial fuel cells (MFCs) and
microbial electrolysis cells (MECs). The MFCs and MECs have the potential to be implemented at industrial scale for energy generation (Santoro et al., 2017), wastewater treatment (Gude, 2016) and for producing valuable chemicals such as hydrogen (Logan et al., 2008), ethanol (Steinbusch et al., 2010) and hydrogen peroxide (Rozendal et al., 2009). Along with the application in energy production processes, EABFs has also been incorporated for the development of biosensors with better selectivity and sensitivity for environmental applications. The MFC sensors consisting an EABF coated electrode have been used for monitoring BOD (Yamashita et al., 2016), assimilable organic carbon (Quek et al., 2015), pollutants (Webster et al., 2014) etc. Certain electroactive bacteria such as Faecalibacterium prausnitzii (Khan et al., 2012) and Enterococcus faecalis (Keogh et al., 2018) has been reported as being able to maintain human gut health by facilitating extracellular electron transfer.

The extensive application of EABFs in various industrial and environmental applications and its involvement in human health urges for more understanding on their metabolism to make BES technologies more efficient and to maintain better human health.

1.1.1.1. Composition of extracellular polymeric substance (EPS) in EABFs
The structure and formation pattern of EABF is similar to that of usual biofilms (Read et al., 2010; van Loosdrecht et al., 2002). The major component of an EABF is its EPS matrix which holds the bacterial cells and their redox active metabolites (electron mediators) or cell components (e.g. nanowires, c-type cytochrome) together to facilitate the electrochemical connection with the electrode. Thus, the function of an EABF depends largely on the structure and composition of its EPS matrix.

Several imaging methods such as confocal laser scanning microscopy (CLSM), atomic force microscopy (AFM), scanning electron microscopy (SEM), epifluorescence
microscopy have been applied to study the structure of an EABF (Bond & Lovley, 2003; Franks et al., 2009; Read et al., 2010; Yi et al., 2009). Thus, more investigation is needed to elucidate the role of the EABF components in assisting electron transfer within the biofilm.

In the context of BES, the structure of an EABF mainly depends on the design and operational factors of the BES and the biofilm thickness can be varied from monolayer to multilayer (Borole et al., 2009; Franks et al., 2009; Read et al., 2010). The viable cell density within the biofilm layers has also been observed to depend on the operational condition and the highest viability was reported on the biofilm-electrode interface (Read et al., 2010).

In anodophilic biofilms, the rate of electron transfer (current density) to the electrode (anode) has been reported to be limited by the accumulation of protons which results in pH decrease. The pH change occurs mainly in the biofilm layer exposed to the electrode where release of electron to the electrode results in the deposition of protons in the biofilm (Franks et al., 2009; Torres et al., 2008). For instance, in one study, one unit drop in pH value was observed between the external medium (pH 7) and anode-biofilm interface (pH 6.1) (Franks et al., 2009). However, other researchers (Babauta et al., 2012) suggested that pH is not always a limiting factor and it depends on growth phases of bacteria. Thus, the effect of current density on the pH and redox potential inside a biofilm still remain controversial (Babauta et al., 2012).

1.1.1.2. Electron flow in biological systems

In nature, life of any biological entity depends upon the flow of electrons (electron acceptance and transfer) and this principle of life established the axiom ‘electrons must flow’ (Nealson & Finkel, 2011). Although there are some exceptions in this rule, in most
cases, flow of electron creates a chemo-osmotic gradient which is required for adenosine triphosphate (ATP) production (Nealson & Rye, 2003; White, 2007). The cessation of this electron flow prevents the cells to generate energy (ATP) which ultimately results in cell death. This phenomenon is very significant in microbial ecology. In nature, redox-active microorganisms could exploit a redox gradient existing in their growth environment and accelerate the electron transfer rate for their own benefit (Nealson & Finkel, 2011).

Electron flow in biological system could be simplified as the flow of electrons from the donor to the acceptor. The electron donor (e.g. organic carbons) and acceptor (e.g. oxygen) for eukaryotic respiration are specific. However, other than organic carbons and oxygen, prokaryotic cells can utilize a wide range of compounds as their electron donors (e.g. hydrogen; H$_2$ (Stams & Plugge, 2009)) and ferrous iron; Fe$^{2+}$ (Ehrenreich & Widdel, 1994)) and acceptors (e.g. NO$_3^-$, SO$_4^{2-}$, Fe$^{3+}$, CO$_2$) to accomplish respiration. The abundance of these molecules in nature supports the growth of different types of microorganism leading to the development of a diversified microbial ecosystem.

It is well accepted that not only soluble compounds can be used as electron acceptors, insoluble compounds such as Fe(III) and Mn(IV) can also serve as electron acceptor for some microorganisms (Beal et al., 2009; Lovley & Phillips, 1986). Depending on the potential of a conductive surface, electricigens are known to be capable of utilizing it as electron donor (cathodic biofilm) or acceptor (anodic biofilm). The electricigens establish an electron flow between the electrode and electron acceptor (cathodic biofilm) or between the electron donor and electrode (anodic biofilm) to accomplish their respiration.
The capability of microorganisms to thrive under different unusual conditions using diverse compounds as their electron donor and acceptor has made microbial EET an interesting topic to explore further.

1.1.1.3. Mechanisms of electron transfer in EABFs
The ability of microorganisms to exchange electrons with an electrode is critical for the development of EABF on an electrode surface. These microorganisms, typically known as electricigens, have been reported to employ three different mechanisms to facilitate exchange of electrons with an electrode. These mechanisms are (1) indirect electron transfer by using exogenous or endogenous electron shuttles or mediators (Brutinel & Gralnick, 2012b), (2) direct electron transfer through c-type cytochrome (Shi et al., 2007) and (3) direct electron transfer via conductive nanowires (Reguera et al., 2005; Strycharz-Glaven et al., 2011).

1.1.1.3.1. Electron transfer by soluble electron shuttles or mediators
The involvement of electron mediators in microbial EET has been extensively studied. A wide range of bacteria were found to operate EET by producing soluble electron mediators (Brutinel & Gralnick, 2012b; Newman & Kolter, 2000; Rabaey et al., 2007). Electron mediators assist the microorganisms to respire on electron acceptors that are not readily available (e.g. insoluble or at low concentration) or toxic to the microorganisms. For instance, it is known that under oxygen limiting condition, Pseudomonas aeruginosa uses pyocyanin (PYO) to transfer electrons to molecular oxygen (Price-Whelan et al., 2007). Some bacteria such as Shewanella onidensis (Lies et al., 2005) and Geothrix fermentans (Nevin & Lovley, 2002) have been shown to rely on the presence of soluble electron mediators for reducing insoluble Fe(III) oxides. The anaerobic human gut
bacteria known as *Faecalibacterium prausnitzii* uses flavin and thiol to utilize oxygen of the epithelial cells as terminal electron acceptor (Khan et al., 2012).

The presence of electron mediators (biofilm produced and externally added) has been reported to enhance the current generation in MFCs due to their efficient electron transfer capability (Lin et al., 2014; Qiao et al., 2017; Rabaey et al., 2005; Sun et al., 2013; Velasquez-Orta et al., 2010). Although the application of electron mediators is known mostly for catalyzing anodic electron transfer, their involvement was also reported for catalyzing cathodic reactions. For instance, an electron mediator (e.g. pirroloquinoline quinone) was found to act in concert with *Acinetobacter calcoaceticus* for oxygen reduction in a MFC biocathode (Freguia et al., 2010).

Involvement of electron mediator has also been reported in microbially influenced corrosion (MIC) of steel. The addition of electron mediators (e.g. FAD and riboflavin) in a steel exposed *Pseudomonas aeruginosa* (Jia et al., 2017b) and *Desulfovibrio vulgaris* (Zhang et al., 2015) was found to increase the corrosion rate. In these MIC studies, the role of the electron mediators was proposed to extract electrons from the steel which resulted in the acceleration of cathodic corrosion reaction. The roles of two biologically important electron mediators are briefly described below:

### 1.1.1.3.1.1. Flavins

Flavins are yellow-coloured redox active organic compounds containing the basic structure of 7,8-dimethyl-10-alkyloalloxazine. In biological system, riboflavin (vitamin B2) serves as the precursor for all other important flavins and it is one of the most extensively studied biological electron mediators (Edwards, 2014). The redox reaction of flavins involve the transfer of one or two electrons for the interconversion between its three redox states namely oxidized, monovalently reduced and divalently reduced (Bailey & Schultz, 2016). Many bacteria have been reported to produce or utilize...
different flavin derivatives such as riboflavin, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) to accomplish their EET processes.

*Shewanella* is one of the well-known flavin producers and has been reported to use this compound as the main electron transfer machinery. The reliance of *S. oneidensis* on flavin’s electron shuttling ability is the most well-established example of EET (Baron et al., 2009; Marsili et al., 2008). It has also been reported that the reduction of ferric iron by *Shewanella* is flavin dependent where flavin mononucleotide (FMN) and riboflavin generates an electron flow between the bacterial cell and Fe (III)-citrate or fumarate (von Canstein et al., 2008). The reduction of flavins in *S. oneidensis* occurs in the outer membrane by a c-type cytochrome (MtrC) that acts as an electron carrier from the periplasm to the outer membrane (Clarke et al., 2011; Hartshorne et al., 2009). Direct electron hopping (transfer of electron between two neighbouring atoms) from MtrC to an electrode has been also reported (Baron et al., 2009; Liu et al., 2010), but was only found to be possible if the anode is poised at a highly positive potential (+0.4 V vs. Ag/AgCl).

Flavins have been reported to play a vital role in the gut microbial system by assisting anaerobic bacteria to respire oxygen. For instance, an important gut bacterium called *Faecalibacterium* was reported to rely on flavins to access oxidation power of oxygen (Khan et al., 2012). Another important gut bacteria *Enterobacter faecalis* was also reported to take advantage from the electron shuttling ability of riboflavin (Zhang et al., 2014). The lower amount of flavin compounds would limit the respiration of these bacteria which will lead to cell death. Thus, the presence of a sufficient amount of flavin compounds are needed for the survival of these bacteria in the gut system. The decreased amount of important gut bacteria (e.g. *Faecalibacterium*) has already been linked to the development of intestinal disorders (e.g. Crohn’s disease) (Sokol et al., 2008).
finding suggests the importance of the presence of electron mediators in supporting a healthy gut microbiota.

1.1.1.3.1.2. Pyocyanin

Pyocyanin (PYO) is a redox active blue-green compound produced by *Pseudomonas aeruginosa* (PA). The redox reaction of PYO involves the transfer of two electrons which results in the interconversion between its oxidized (blue-green) and reduced (colourless) form (Bellin et al., 2016). However, PYO also has been reported to exist in a monovalently reduced state (Goyal & Manoharachary, 2014).

The purpose of PA to produce PYO has been reported to assist in transferring electrons to oxygen where the diffusion of oxygen is limited inside the biofilm (Dietrich et al., 2008; Price-Whelan et al., 2007). However, being an aerobic organism, it is not reasonable for PA to use mediator to respire on oxygen. Besides, the air exposed biofilms of PA should not be limited by oxygen. Although some studies reported the existence of anaerobic zone within the PA biofilm produced on the lung of cystic fibrosis patients (Hassett et al., 2002), PA cells in that zone was reported to respire on nitrate. Thus, the use of PYO as a shuttle for PA to respire on oxygen by air exposed PA biofilm seems not logical.

PYO can penetrate inside the living cells and has been reported to oxidize the cellular NADH (Britigan et al., 1992). The reduced PYO thus generated, is believed to react with oxygen to generate reactive oxygen species (ROS) such as super-oxides and peroxides. The generation of ROS compounds increase the oxidative stress inside the cell leading to cell death. (Gloyne et al., 2011; Muller, 2006). PA has been reported to operate this PYO mediated mechanism to destroy their host cells (Hassan & Fridovich, 1980). This host cell destruction could be beneficial for PA to use the damaged cells as the source of
organics. This study investigates the possibility of PYO to offer any other benefits to its producing bacteria (PA).

The electron mediating role of PYO has also been investigated in MFC where the externally added PYO accelerated the current generation both by PA and microorganisms other than PA (Rabaey et al., 2005). Under substrate rich condition, the reduction of PYO by PA to transfer electrons to the anode is expected. However, for non-PA cells it would be interesting to investigate whether PYO reduction is deliberately performed by these cells or PYO with its cell penetrating ability takes the electrons (by oxidizing NADH) out of the cells.

All the above-mentioned roles of different electron mediators in microbial EET encourage further investigation on the specific role of these redox active compounds.

1.1.1.3.2 Direct Electron transfer by redox active protein

The involvement of redox active proteins like c-type cytochromes has been reported for short range electron transfer by microorganisms (Bond & Lovley, 2003; Chaudhuri & Lovley, 2003; Marshall & May, 2009; Wrighton et al., 2011). This mode of electron transfer is very common in *G. sulfurreducens* which predominate on anodes and transfers electrons from organics to the electrode (Kiely et al., 2011; Lovley et al., 2011). Outer membrane of *G. sulfurreducens* contains most of their diverse c-type Cytochromes (Inoue et al., 2011; Mehta et al., 2005). The purified outer membrane c-type cytochromes can reduce known extracellular electron acceptor in vitro (Inoue et al., 2011; Qian et al., 2007). Similar reduction property was also found in vivo by applying gene deletion method (Mehta et al., 2005; Shelobolina et al., 2007; Voordeckers et al., 2010). In most *G. sulfurreducens* biofilm, the attachment of cytochromes in the electrode proximity creates an electrochemical gate for direct electron transfer to the electrode (Busalmen et
Gene expression and deletion method is so far, the most widely used method to determine the role of several outer surface c-type cytochrome in electron transfer (Holmes et al., 2006; Nevin et al., 2009). Among various surface c-type cytochromes, a hydrophobic protein having the molecular mass of 30 kDa and midpoint potential -0.22 V called OmcZ, treated as the major cytochrome in high current producing biofilms (Inoue et al., 2011; Nevin et al., 2009). Interestingly, it has recently been reported that direct electron transfer by c-type cytochrome also needs the involvement of the electron mediator to link between cytochrome and electron acceptor (Okamoto et al., 2014).

1.1.1.3.3. Electron transfer by microbial nanowires

Bacterial or bacterial nanowires are mainly produced by metal reducing bacteria such as Geobacter sulfurreducens and Shewanella oneidensis and suggested to be used as an effective long range extracellular electron transfer apparatus (Gorby et al., 2006; Reguera et al., 2005).

Bacterial nanowires are conductive appendages which was believed to be composed of pilin proteins, mainly comprises of the subunit called PilA (Arts et al., 2007; Craig et al., 2006). This belief was supported by a study where a PilA mutant of G. sulfurreducens strain with Aro-5 lacking aromatic residues showed lower conductivity than that of the wild one (Vargas et al., 2013). In contrast to this belief, the conductivity of nanowires has been linked to c-type cytochrome and it was found that S. oneidensis mutants deficient in MtrC and OmcA gene (genes for c-type cytochrome) produces nanowire like structures which are non-conductive (El-Naggar et al., 2010). Recently, bacterial nanowires have been proposed as an extended form of outer membrane and periplasm consisting of multiheme cytochromes rather than pilin proteins (Pirbadian et al., 2014).
Although the use of bacterial nanowire in bridging the electron transport chain and external electron acceptor is a very interesting concept, more investigation is still needed to know the actual composition and mode of electron transfer within nanowires (Pirbadian et al., 2014).

Nanowires have been reported to transfer electrons up to micrometre length with the electron transfer rate of $10^9$/second between 0.1 V potential difference (El-Naggar et al., 2010). Two mechanisms known as electron hopping and metal like conductivity (MLC) have been proposed to explain the electron conduction through nanowires (Malvankar et al., 2011; Strycharz-Glaven et al., 2011). However, the belief of nanowire as a pilin (non-conductive) polymer creates controversy on the proposed electron transport mechanisms. The latest finding on the composition of nanowire where the structure of nanowire was reported to be composed of multiheme cytochromes (Pirbadian et al., 2014), is supportive to the MLC which is so far, the most acceptable theory of electron conduction within the nanowires.

The anodic chamber of a BES supports the growth of both planktonic and sessile cells (in biofilm) capable of performing extracellular electron transfer to the electrode. Mediator dependent electron transfer either endogenous (bacterial self-produced) (Pham et al., 2008) or exogenous (externally added) (Ieropoulos et al., 2005; Park et al., 1999) is the most common type of microbial EET process. A few evidences were found for direct electron transfer with c-type cytochromes or nanowire mediated electron transfer (Biffinger et al., 2007; Bond & Lovley, 2003; Reguera et al., 2006). Preference of electron transfer mechanism varies between Planktonic and sessile bacterial cells. The preferred mode of electron transfer by planktonic cells is mainly using mediators whereas sessile biofilm bacteria can respire on anode by employing any of the three above mentioned mechanisms (Yang et al., 2015). It is believed that a better electron transfer
kinetics can be achieved in direct or nanowire supported processes rather than with electron mediators where the slow diffusive flux rate could slow down the electron flow rate (Lovley, 2006; Marsili et al., 2010; Sрикант et al., 2008).

1.1.1.4. Interspecies electron transfer (IET)

IET is the electron exchange mechanism between the cells of different species which enable them to gain energy under a condition where a single species unable to catalyze the reaction. Interspecies electron transfer can be categorized into two types namely mediated interspecies electron transfer (MIET) and direct interspecies electron transfer (DIET).

MIET is one of the earliest proposal on the mechanism of electron transfer between two organisms where the electrons are proposed to be transferred by exchanging metabolites between them. For example, it was believed that the conversion of ethanol into methane (CH$_4$) can be performed by methanogens alone. However, later, it was reported that the conversion of ethanol into CH$_4$ is not accomplished only by methanogens and accompanied by another bacterium (Bryant et al., 1967; Johns & Barker, 1960). The mechanism involves the fermentation of ethanol by the bacterium to generate hydrogen (H$_2$) which subsequently utilized by the methanogen as the electron donor to reduce CO$_2$ to form CH$_4$ (Bryant et al., 1967). MIET also called interspecies hydrogen transfer and can be observed in different microbial systems like anaerobic digesters, landfills and marine sediments (Heimann et al., 2006; Ishii et al., 2005; Stams et al., 2006).

DIET is comparatively the latest concept on the electron transfer mechanism between microorganisms (Summers et al., 2010). According to this mechanism, electrons are transferred from one cell to another directly through the conductive channel created by the physical contact of membrane-bound structures (McGlynn et al., 2015; Wegener et
al., 2015). Unlike to the diffusion of metabolites (as electron sources) in MIET, DIET involves the direct electron flow between two cells through the conductive bridge formed by membrane-bound components, mainly cytochromes and pili (Cheng & Call, 2016). However, externally added conductive materials such as zero valent iron (Wu et al., 2015), magnetite (Cruz Viggi et al., 2014), graphite (Zhao et al., 2015), biochar (Lü et al., 2016) has also been found to enhance the electron transfer rate between cells.

DIET has been reported to be an effective mechanism for syntrophic relationship in methanogenic communities. For instance, the anaerobic oxidation of methane involves the action of anaerobic methanotrophic archaea (ANME) in concert with sulfate-reducing bacteria (SRB) (Wegener et al., 2015). Under the experimental condition, overexpression of the genes for cytochrome were observed in both bacteria which were connected by nanowire like structure. Nanowire like appendages were found in SRB only when ANME was their only electron source. Interestingly, the availability of hydrogen stopped the formation of nanowire like filaments in SRB. This finding indicates the necessity of conductive appendages for interspecies electron transfer.

1.1.1.5. Impact of EABFs

1.1.1.5.1. EABFs in human gut
The gut microbiome is one of the emerging research areas in recent years which has been considered to have direct influence in regulating human health (Balskus, 2018; Koppel et al., 2017; LeBlanc et al., 2013). Human intestine is a reservoir for a diverse group of microorganisms that would affect the well-being of human. Large intestine is the site which is heavily colonized with diverse group of bacteria and microscopic images showed that bacteria in this part of the gastrointestinal (GI) tract are mainly exist as microcolonies or in association with other bacteria.
Gut microorganisms are treated either as causative agents or maintenance factors responsible for many bowel disorders. However, the causative agents of most of the diseases associated with large intestine are still unknown (Macfarlane & Dillon, 2007). Recently, elucidating the relationship between GI tract bacteria and disease development has been a subject of research. For instance, one study found that Crohn’s disease was associated with *Faecalibacterium prausnitzii* which is an important butyrate supplier to the colonic epithelium and are oxygen sensitive (Khan et al., 2012). However, it is interesting that how this oxygen sensitive bacterium colonizes in the gut mucosa where oxygen diffuses from the epithelial cell. Researchers claimed that this bacteria can utilize oxygen as a terminal electron acceptor and transfer electrons through producing electron shuttles or mediators like flavin or thiol compounds which are abundant in healthy human gut (Khan et al., 2012). This research suggests flavin or other antioxidant rich diets could be the remedy for Crohn’s disease.

Similarly, there would also be a chance of the existence of other bacteria in the gut which may be utilizing the redox gradient between the inner intestinal lining (anaerobic) and upper epithelial cell (oxygenated). The gut microbiome thus has a correlation with human health and understanding their metabolic electron transfer mechanisms will help to formulate remedy for various gastrointestinal disorders.

### 1.1.1.5.2. Role of EABFs in MFC

The concept of MFC technology was proposed as a promising way to produce electricity from waste (Rabaey & Verstraete, 2005). Although MFC is regarded as a green technology, the slow electron transfer rate in both anodic (Torres et al., 2008) and cathodic (Rismani-Yazdi et al., 2008) chamber is considered as one of the major limitations preventing this technology to work with optimum efficiency.
In MFC, EABF oxidizes the substrates present in anolyte and then act as electron donor for the anode. To investigate the role of these bacteria in anodic chamber, different electroactive bacteria was isolated by enriching them in the presence of artificially poised anode electrode so that it could accept electrons from the bacteria (McLean et al., 2010; Torres et al., 2009). Though the most commonly known property of EABF was their ability to transfer electrons to solid conductive surfaces, it is now apparent from several studies that some bacteria can also consume electrons from electron rich electrodes (Rabaey & Rozendal, 2010). Application of this type of bacteria in cathodic chamber facilitates better oxygen reduction reaction due to their high oxygen affinity. Interestingly, one study recently revealed the existence of a single biofilm which can catalyze both anodic oxidation and cathodic reduction reaction and named anocathodophilic biofilm (Cheng et al., 2012). This biofilm was used as both bioanode and biocathode for acetate oxidation and denitrification, respectively with more than 85% coulombic efficiency.

In nature, the role of this type of bacteria is very important in biofilms where cells remain in an intimate contact with each other between a strong redox gradient. If a single species has both electron donation and uptake capability, the flow of electrons through their cell is advantageous to the whole biofilm community. For maintaining a sustainable growth on a conductive surface, biofilm bacteria should be specialized in electron uptake and transfer. For instance, a biofilm growth on an electron acceptor would be sustainable if the bacteria exposed to the electron acceptor are highly active in electron transfer and those exposed to electron donor are specialized for electron uptake (Nealson & Finkel, 2011).
1.1.5.3. Microbially Influenced Corrosion (MIC)

Metal corrosion can be simplified as the oxidation of zero-valent metal in the presence of strong oxidizer such as oxygen which takes electrons away from the metal (cathodic site) resulting in the dissolution of metal from the anodic site (Hamilton, 1985). Metal corrosion has also been reported to occur in the absence of oxygen where microorganisms catalyze the cathodic electron transfer from the metal to the electron acceptors such as nitrate and sulfate (Cord-Ruwisch & Widdel, 1986; Jia et al., 2017b; Venzlaff et al., 2013). The corrosion which is initiated or accelerated by the microorganisms is known as microbially influenced corrosion (MIC).

The role of biofilms on anaerobic MIC has been extensively studied and sulfate or nitrate reducers was considered as the major anaerobic corrosion causing bacteria. In this process, the bacteria establish an electron flow by utilizing metal as the electron donor and sulfate or nitrate as the terminal electron acceptor (Beese-Vasbender et al., 2015). This electron flow causes electron loss from the metal (cathodic stimulation) which as a consequence, dissolves the metal ions (Kakooei et al., 2012; Mand et al., 2014). However, recently a synergistic interaction between Desulfovibrio and homoacetogens was reported to involve anaerobic MIC where carbon dioxide (CO$_2$) was present as the electron acceptor instead of sulfate (Usher et al., 2015). According to this study, Desulfovibrio transfers the electrons from the metal to Acetobacterium which reduces CO$_2$ as the electron acceptor and produce acetate to provide carbon source for Desulfovibrio.

The role of microorganisms in influencing aerobic corrosion is still debatable. Some studies suggested the role of aerobic biofilm as corrosion inhibitor (Chongdar et al., 2005). However, others reported an indirect influence of this biofilm in enhancing corrosion. The aerobic biofilm has been reported to reduce EPS bound Fe$^{3+}$ which was
formed due to the oxidation of Fe$^{2+}$ (dissolved form the anodic site) by oxygen. This biofilm assisted flow of electrons from the steel to Fe$^{3+}$ will re-generate Fe$^{2+}$ which would then be re-oxidized by oxygen. Thus, the electrons from the steel would ultimately be transferred to oxygen which was proposed to increase the corrosion rate (Beech & Sunner, 2004). However, no study has been conducted yet to investigate the role of bacteria or their redox active metabolites in influencing aerobic corrosion.

The role of cathodophilic biofilm in enhancing oxygen reduction reaction (ORR) has been reported in one study (Bergel et al., 2005). However, the associated mechanism for such efficient reduction reaction was not evaluated. Microbial electron mediators such as FAD and riboflavin have been employed both individually and in association with bacteria (PA and SRB) to monitor their effect on steel corrosion under anaerobic condition (Jia et al., 2017b; Zhang et al., 2015). Although the presence of these mediators with PA didn’t cause significant increase in corrosion rate, this finding encourages the investigation on the effect of PYO (PA produced electron mediator) in the corrosion rate of a PA exposed steel surface.

1.1.2. Techniques available to evaluate electroactive biofilms

Bioelectrochemistry deals with the study and application of biological electron transfer processes using different electrochemical analysis such as voltammetry, amperometry and conductometry. Typically, these techniques are conducted with a three-electrode electrochemical system, consists of working, reference and counter electrode. In this setup, the potential of the working electrode (WE) is controlled with respect to the reference electrode (RE) using a potentiostat where counter electrode (CE) serves as the auxiliary electrode.
There are different types of techniques which can be used to analyze the electrochemical signals generated by an electroactive biofilm. However, three electrochemical techniques are commonly used in most studies namely amperometry, voltammetry and conductometry.

1.1.2.1. Amperometry
Amperometry is an electrochemical analytical technique in which current production in an artificially poised electrode is recorded as an analytical signal where electron flow is generated due to oxidation or reduction (Ding et al., 2008; Roussel et al., 2008). When a molecule is oxidized at the electrode surface, electron flows from the molecule to the electrode whereas during reduction, electron flows in the opposite direction. This is an efficient method for understanding the relationship between the electrode potential and the current. With this method, one could obtain steady state current (activity) of the biofilm at various fixed electrode potential (e.g. polarization curve). Amperometric techniques are mostly applied to monitor the generation of current in microbial fuel cells and also to analyze the signals in electrochemical biosensors.
1.1.2.2. Voltammetry
 Voltammetry is the most common electroanalytical technique used in bioelectrochemistry where the electrochemical property of an analyte is determined by monitoring the current over the voltage change. Typically, potential of an electrode is controlled by an electronic instrument called potentiostat. However, in some cases, voltammetry can also be performed without using a potentiostat (Cheng et al., 2009). A potentiostat usually consists of three electrodes namely the working (WE), reference (RE) and counter (CE) electrodes. WE is the electrode under controlled potential where current is measured and usually made of inert conductive metals such as gold, platinum, carbon etc. RE has a fixed potential and the WE potential is measured against this electrode. Potentiostat maintains a certain potential between working and reference electrode by sucking or depositing electrons from the CE through the cell and measures electron flow between working and counter electrode as current.

Figure 1.1. A amperometric plot generated during the development of an anodic biofilm on Indium tin oxide (ITO) at -0.3 V with 10 mM acetate as the electron donor.
It is a very useful tool to evaluate the properties of electricigens and their biofilms. Electron transfer rate, electron transfer mechanism, detection and evaluation of the electron mediators can be done by applying different voltammetric techniques (e.g. Cyclic voltammetry, Differential pulse Voltammetry, AC voltammetry etc.).

1.1.2.2.1. Cyclic voltammetry
Cyclic voltammetry (CV) is the most widely used technique for qualitative evaluation of electrochemical reactions (Heinze, 1984). This is a potential sweeping method in which potential of an electrode is changed by a specific sweep rate (mili volt/sec) to facilitate oxidation and reduction of the target molecules in a cyclic order. The current generated is then plotted against the corresponding potentials and the obtained figure is termed as cyclic voltammogram (Cournet et al., 2010). In BES, CV is widely applied to study electron transfer between the bacteria or EABFs and the electrode (Busalmen et al., 2008b; Rabaey et al., 2004). The mid-point of an electron mediator present on the boundary layer of the WE can indirectly be determined by CV (Baron et al., 2009). It is also an effective way to get information about different growth phases of bacteria and researchers found many aspects of EABFs growth stages like substrate utilization, electron transfer rate or mechanism by applying CV in a bioelectrochemical system (Fricke et al., 2008). However, the data achieved by applying CV is not enough to understand the complex electrotrochemical processes in an EABF. Combination of information from other bio-analytical approaches such as molecular biology and metabolomics could make CV more efficient bioelectrochemical analysis tool (Fricke et al., 2008).
1.1.2.2. Differential pulse voltammetry

Differential pulse voltammetry (DPV) is another widely used approach for electrochemical analysis (Ensafi et al., 2010). This process starts with an induction period where the electrochemical cell is operated under initial conditions to allow the system to stabilize. Thereafter, the potential of the working electrode is changed by a certain potential step (pulse) to perform forward and backward steps. The increment in the potential for each forward step is kept constant and the differential current for each step (forward and backward) is plotted as a function of the potential applied. DPV only extract Faraday current thus it is easy to evaluate the actual reaction of the electrode. Moreover, being a sensitive technique, DPV is very effective for studying the redox properties of chemicals even if present in trace amounts.
1.1.2.2.3. AC voltammetry

AC Voltammetry involves the application of a sinusoidal oscillating potential to an electrochemical cell. The current generated for each potential change is recorded and plotted as a function of corresponding applied voltage. This technique is used along with locking amplifier or frequency analyzer which offers improved sensitivity than other traditional voltammetry techniques. This is a useful analytical technique in a diffusion driven (mode of transport) electrochemical cell (Laviron, 1979).

1.1.2.3. Conductometry

Conductometry is the technique to evaluate the conductivity of a solution. The variations in the conductivity of a solution occurs due to the changes in ion concentrations such as during microbial metabolism (Lei et al., 2006; Shul'ga et al., 1994). The fastness, sensitivity and easy miniaturization (no need for reference electrode) property (Shul'ga et al., 1994) make conductometric biosensors as an attractive choice (Mikkelsen & Rechnitz, 1989).

In conventional electrochemical studies, the redox properties of the target molecule in the boundary layer of the WE are evaluated for its characterization. Since the existing three electrode system have only one WE, it allows either oxidation or reduction at a time. However, in microbial systems, the oxidation and reduction reaction occur simultaneously. Thus, the conventional electrochemical system may not be ideal for evaluating microbial electron transfer processes. This limitation creates a scope for the improvement of conventional setup to develop a more efficient electrochemical device for evaluating microbial EET processes.
1.1.3. Significance of studying electroactive biofilm

The understanding of the properties of electroactive biofilms and their EET processes is important to develop efficient BES technologies (e.g. MFC, biosensor, bioremediation etc.) and control bioprocesses which are detrimental (e.g. metal corrosion) or harmful (e.g. Crohn’s disease) from human health perspective.

The concept of microbial fuel cell was emerged as an attractive way to generate electricity from the waste relying on the oxidation of organics by electricigens and subsequent deposition of electrons to an electrode. However, this technology is still facing obstacles such as cathode limitation, ohmic loss, mass transport loss and substrate cross over (O'Hayre et al., 2006) which are preventing MFC to work with optimum efficiency. Further investigation on the anodic and cathodic MFC biofilms for improving their electron transfer capability would make this green technology feasible for commercial implementation.

MIC of metal is one of the major global concern affecting many industry and communal services where electroactive biofilms corrode metals by extracting electrons from them. It has been known that under anoxic environment (e.g. in gas or oil pipeline), sulfate and nitrate reducing bacteria play the key role in MIC. The proposed mechanisms involve in this process are catalyzing an enhanced cathodic reaction, thus acts as cathodic stimulant or producing corrosive chemicals like hydrogen sulphide (Enning & Garrelfs, 2014). Recently, researchers have found that SRB is not the only bacteria that cause steel corrosion but it is the result of syntrophic interaction of different groups of microorganisms along with SRB such as sulphur reducing bacteria (SRB) and acetogens (Usher et al., 2015). The possibility of the involvement of interspecies electron transfer during MIC is an interesting concept that warrants further research. It is known that cathodophilic bacteria are capable of overcoming the over-potential of oxygen to
facilitate oxygen reduction reaction (Bergel et al., 2005). This finding could be linked to the investigation on the potential role of electroactive biofilm in enhancing aerobic metal corrosion.

Human gut biofilm has been reported to have a direct impact on human health, could be the reason for the axiom “You are what you eat”. For instance, metabolites of gut bacteria e.g. butyrate, acetate and flavins found to have direct impact on immune system (Arpaia et al., 2013) and these metabolites also prevent the colonization of pathogenic bacteria (Sun & O’Riordan, 2013; Ubeda et al., 2017). However, only little is known about the interaction among the gut microbial flora and the causes responsible for developing intestinal disorders.

Overall, the electroactive biofilms play a significant role in various natural and engineered environments which are linked to human interest. Hence, extensive research on the above-mentioned electroactive biofilm associated technologies and processes would create scopes to modify the existing situation to control the biofilm activity according to the requirement.
1.2. Aim and scope of the thesis

The aim of this thesis is to evaluate the role of biological electron mediators in microbial EET processes and this research program can be broadly divided into four major concepts:

1. Developing a novel electrochemical cell to overcome the limitations of conventional three-electrode system in evaluating microbial electron transfer processes. (Chapter 2)

2. Proposing an improved method to characterize redox properties of electrochemically active molecules. (Chapter 2)

3. Investigating the electron transfer capabilities of an important bacterial electron mediator (pyocyanin) to re-evaluate its role in the interaction of PA cells with other living cells and its significance in PA infections. (Chapter 3)

4. Evaluating the role of pyocyanin in influencing aerobic microbial corrosion by electron extraction from rusting steel. (Chapter 4)
Chapter 2: New method for characterizing electron mediators in microbial systems using a thin-layer twin-working electrode cell

Abstract
Microbial biofilms are significant ecosystems where the existence of redox gradients drive electron transfer often via soluble electron mediators. This study describes the use of two interfacing working electrodes (WEs) to simulate redox gradients within close proximity (250 μm) for the detection and quantification of electron mediators. By using a common counter and reference electrode, the potentials of the two WEs were independently controlled to maintain a suitable “voltage window”, which enabled simultaneous oxidation and reduction of electron mediators as evidenced by the concurrent anodic and cathodic currents, respectively. To validate the method, the electrochemical properties of different mediators (hexacyanoferrate, HCF, riboflavin, RF) were characterized by stepwise shifting the “voltage window” (ranging between 25 and 200 mV) within a range of potentials after steady equilibrium current of both WEs was established. The resulting differences in electrical currents between the two WEs were recorded across a defined potential spectrum (between –1 V and +0.5 V vs. Ag/AgCl). Results indicated that the technique enabled identification (by the distinct peak locations at the potential scale) and quantification (by the peak of current) of the mediators for individual species as well as in an aqueous mixture. It enabled a precise determination of mid-potentials of the externally added mediators (HCF, RF) and mediators produced by pyocyanin-producing Pseudomonas aeruginosa (WACC 91) culture. The twin working electrode described is particularly suitable for studying

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Chapter 2. New method for characterizing electron mediators using a thin-layer twin-working electrode cell

mediator-dependent microbial electron transfer processes or simulating redox gradients as they exist in microbial biofilms.

2.1. Introduction

Electroactive bacteria can transfer electrons to insoluble electron acceptors via different mechanisms such as using exogenous or endogenous electron shuttles or mediators (Brutinel & Gralnick, 2012a), direct electron transfer through outer membrane c-type cytochromes (OM c-Cyts) (Shi et al., 2007) and production of conductive nanowires (Bond et al., 2012; Reguera et al., 2005). Arguably, mediator dependent electron transfer is the most common mode of electron transfer, where a wide range of bacteria accomplish extracellular electron transfer (EET) by producing various soluble electron shuttles or mediators (Rabaey et al., 2007). For instance, it is well known that the distal Fe (III) reduction capability of Shewanella species is dependent on their ability to excrete flavin molecules (Baron et al., 2009; Lies et al., 2005; Marsili et al., 2008; von Canstein et al., 2008). Respiration of Geothrix on insoluble Fe(III) oxides was also reported to be soluble electron shuttle dependent (Nevin & Lovley, 2002). Recently, it has been reported that the direct electron transfer of Geobacter also required flavin molecules to mediate electron transfer between the heme group of OM c-Cyts and the electrode (Okamoto et al., 2014). In fact, the role of mediators in EET has been well-known in microbial fuel cell (MFC) and microbially induced corrosion (MIC) research where the mediators were often shown to enhance current production (Lin et al., 2014) and accelerate corrosion rate (Li et al., 2015a; Zhang et al., 2015), respectively. Along with flavins, some other compounds such as anthraquinone-2, 6-disulfonic acid (AQDS) (Sun et al., 2013), cysteine (Doong & Schink, 2002) and quinone (Freguia et al., 2009) have also proved their electron transfer capability.
Given their important role in EET, it is crucial to characterize and quantify soluble electron mediators in the relevant microbial systems. Currently, various amperometric and voltammetric techniques have been widely used to detect and evaluate electrochemical properties of redox active entities, including soluble mediators (Dryhurst, 2012; Roussel et al., 2008). These conventional techniques are typically carried out by using three-electrode systems, whereby oxidation or reduction of the target compound present in the boundary layer of the working electrode (WE) can be precisely controlled and/or monitored (Compton et al., 2013). In particular, cyclic voltammetry (CV) has been the most common technique, as it enables determination of kinetic and thermodynamic information of a redox system by varying the scan rate and the potential of a single working electrode (Bai et al., 2010; Fricke et al., 2008; Marsili et al., 2010; Sumathi et al., 2015). However, the use of a single working electrode in these conventional settings only enables the record of either anodic or cathodic electron flow at a particular time point during the analysis. It also does not enable the evaluation of the electrochemical behavior of those compounds present beyond the WE’s boundary layer (Bard & Faulkner, 2001). Since both electron accepting (anodic) and electron donating (cathodic) reactions may concurrently occur within close proximities where EET prevails (Malkin et al., 2014), using a single WE for the evaluation of electron mediators in microbial systems may not be ideal.

In this study, we developed a new electrochemical method for evaluating electron mediators in small (μL)-scale aqueous systems. The method involves the use of two closely mounted, but independently controllable WEs to facilitate simultaneous measurement of both electron accepting (anodic) and donating (cathodic) reactions within a thin-layer system (here 250 μm). As such, a redox gradient (potential difference) between the two WEs (here, termed as “Twin-WE” system) can be precisely controlled.
over a range of electrode potentials, and the resulting currents independently recorded at
the two WEs can be used to detect soluble electron mediators within the system.
Compared with conventional CV, such approach may be more suitable for characterizing
the redox behaviour of a microbial system. The aim of this study was to evaluate and
demonstrate the suitability of this method for qualitative and quantitative sensing of
soluble biological electron mediators.

2.2. Materials and Methods

2.2.1. Construction of the Twin-WE system

Two electronically isolated potentiostats (manufactured by Murdoch University
Electronic workshop) were combined to establish the electrochemical device used in this
study. Each potentiostat enabled an independent control of one of the two juxta-
positioned WEs (i.e. Twin-WE) located within a thin aqueous layer (250 μm). The
material of the WEs was either gold-coated or indium tin oxide (ITO) coated glass slide
(dimension 75 mm ×25 mm ×1.1 mm and resistance 8-12 Ω/sq; RileySupplies,
Richmond Hill, Canada). The two potentiostats shared a common counter electrode (CE)
(4 mm in diameter and 105 mm in length) and a silver-silver chloride (Ag/AgCl)
reference electrode (MF-2052, BASi, USA) (Figure 2.1). All potentials mentioned in this
article are quoted against Ag/AgCl reference electrode (+0.210V vs. the standard
hydrogen electrode, SHE).
Figure 2.1. Schematic of a Twin-WE system and an anticipated redox reaction mechanism. (Legends: PS=Potentiostat, RE=Reference electrode, CE= Counter electrode, e=electron, R= reduced mediator, O= oxidized mediator. Both counter and reference electrodes were kept 1 centimeter away from the two Indium tin oxide (ITO) glass slides serving as the working electrode.)

A WE chamber was constructed by placing a nonconductive rectangular reverse osmosis membrane frame spacer (0.25 mm thick, 55 mm length, 25 mm width) between the two conductive surfaces of the WE (mounted with adhesive tape). This configuration allowed the conductive side of both WEs to be positioned at a fixed distance (250 µm). As such, the boundary layers of the two WEs could be housed in a commonly shared, thin-layer aqueous environment with a maximum working liquid volume of 304 µL. The WE chamber, CE and the reference electrode were immersed in a 50 mL bulk sample solution within a Perspex container (length 8 cm, height 4 cm, width 2.5 cm) (Figure 2.1). The electrochemical system was automated and monitored using a personal computer interfaced with a LabVIEW™ software program (National Instrument™) and a data acquisition card (Labjack™ U12).
2.2.2. Working principle of the Twin-WE system for mediator detection

Mid-potential (MP) of an electron mediator is defined as the potential at which the oxidized and reduced forms of the mediator are present at equal concentration (50:50) (Bard & Faulkner, 2001; Bard et al., 1980). When the potential of the WE is higher (more positive) than the MP, anodic current could be recorded as a result of an oxidation of the reduced mediators. When the potential of the WE is lower (more negative) than the MP, cathodic current could be recorded as a result of a reduction of the oxidized mediators.

In the Twin-WE system, both WEs can be poised at a fixed potential gradient (or “voltage window”) across a range of defined potentials. When such “voltage window” is maintained at either beyond or below the mediator’s MP, short-lasting current is expected as both WEs will only enable either oxidation or reduction, leading towards exhaustion of reactants (reduced or oxidized mediators). However, a continuous and stable current between the two WEs should be attainable when one of the WEs is poised at a potential beyond and the other one below the mediator’s MP. As such, the former one will enable an oxidation (i.e. anodic current), whereas the latter one will enable a reduction (i.e. cathodic current). Hence, the maximal steady-state current between the WEs should occur when their potentials are oppositely (one at oxidizing and one at reducing) deviated from the mediator’s MP by an equal value. Such electrical flow path is closed by the diffusion of mediator and the overall electroneutrality of the system is maintained by the diffusion of ions. In other words, as one electrode continuously oxidizes (WE1), while the other reduces (WE2) the mediator, there is also a net electron transfer from the reducing to the oxidizing electrode within the common bulk solution via diffusion of mediator and counter ion (Figure 2.1).

Based on this principle, the presence of electron mediators between the WEs and their respective MPs can be identified and quantified by maintaining a defined “voltage
window” within a range of potential (e.g. ±1V). The resulting current difference between the two WEs can be plotted against the corresponding mid-point of the “voltage window”. In this study, it was hypothesized that with this plot, the presence of electron mediators could be characterized and quantified based on the peak position and signal magnitude of the current difference.

2.2.3. General operation and detailed experimentation with the Twin-WE system
To begin with an electrochemical analysis, the liquid sample was first loaded (via capillary suction created by the two closely packed WEs) into the WE chamber. This was done by immersing the chamber into a sample solution. Thereafter, all the edges of the WE chamber were immediately sealed with molten agar (1.5%) (with a 1-mL disposable syringe). The agar was allowed to solidify under ambient condition (within approximately two minute). This step was to retain the mediators in the WE chamber, as well as to minimise air (oxygen) intrusion from the supporting bulk electrolyte into the WE chamber. It also enabled ionic flux between the WE and CE.

2.2.3.1. Electron mediators
Hexacyanoferrate (HCF, Fe(CN)₆³⁻) and riboflavin (RF) were used as model electron mediators. The mediators were dissolved in phosphate buffer (100 mM, pH 7.3) to obtain their respective standard solutions with different concentrations. An active culture of Pseudomonas aeruginosa (WACC 91) known to produce pyocyanin (characteristic blue-greenish coloured mediators) was used as a surrogate for a redox active microbial system. The culture was pre-grown in a Pseudomonas Minimal Medium (PMM), which contained (L⁻¹ in deionized water): glycerol (15 mL), L glutamine (5 g), K₂HPO₄ (1.5 g), MgSO₄ (0.2 g) and was harvested 72 hours after sub-culturing. When testing the use of the Twin-WE system for mixed mediators detection, predefined amounts of HCF and RF
standard solutions were mixed with the *P. aeruginosa* culture (25 mL) before being suction-loaded into the WE chamber as described above.

### 2.2.3.2. Cyclic voltammetry (CV) with the Twin-WE system

The electrochemical interaction between the ITO-coated glass slide and the model mediators was first validated by running a conventional CV using the Twin-WE system. Only one of the two WEs was potentiostatically controlled as per conventional practice. CV of HCF was performed at a scan rate of 0.02 V s\(^{-1}\) between +0.7 V and -0.7 V, whereas CV for riboflavin (RF) was done at a lower scan rate of 0.001 V s\(^{-1}\) between 0V and -0.95V. CV for the active *P. aeruginosa* culture and its mixture with HCF and RF was performed at a scan rate of 0.001 V s\(^{-1}\) between +0.85 and -0.95V.

### 2.2.3.3. Shifting a fixed “voltage window” across a potential range with the Twin-WE system

The new method was first validated with a single mediator (HCF) system (1 mM). A series of “voltage window” (25, 50, 75, 100, 125, 150, 175 and 200 mV) was tested to select a suitable window for subsequent tests. Each voltage window was allowed to shift between -0.15V and +0.4V. The currents recorded at the two WEs were allowed to reach a steady state before the voltage window was shifted to the next set point. The time required to reach such steady state was typically within two minutes (data not shown). Hence, a sufficient duration of four minutes was given for each step. The voltage window was shifted (either downward or upward) to the next set point by one quarter of the window’s width (e.g. 12.5 mV/step was given for a 50 mV window). The resulting current difference between the two WEs was plotted against the corresponding mid-point of each voltage window set point. The same approach was used with the selected voltage window for the validation of other mediator systems.
2.2.4. Use of the Twin-WE system as a coulomb counter

Since the aqueous system between the Twin-WE was sealed, the charge storing capacity of the mediators in the WE chamber should in principle, be directly proportional to the amount of mediators present and may thus be quantified electrochemically. To validate this, a sequential charging (reduction)- discharging (oxidation) (i.e. coulomb counting) experiment was conducted. Different concentrations of HCF were precisely loaded to the WE chamber. The theoretical coulomb in the system was calculated based on equation 1.

\[
\text{Theoretical Coulomb} = \frac{(V \times C \times F \times n)}{1000} \quad \text{........... (1)}
\]

Where,
- \( V \) = sample volume inside the WE chamber (L),
- \( C \) = HCF concentration in the sample (mmol/L),
- \( F \) = Faraday constant (96485 C/mole e\(^{-}\)) and
- \( n \) = number of electrons involved in the redox reaction (1 mole of electron per mole of HCF) (reactions 1)

\[
\text{Fe(CN)}_6^{3-} + e^- + H^+ \rightarrow \text{Fe(CN)}_6^{4-} \quad \text{reaction 1) (MP = +0.23V vs. Ag/AgCl)}
\]

(H.L.Kwong, 2004)

After that, complete oxidation and reduction of the mediators inside the WE chamber was performed by maintaining both WEs at either an oxidizing (150 mV beyond the MP of HCF, i.e. +0.3625V) or a reducing (150 mV below the MP of HCF, i.e. +0.0625V) potential. In each case, the currents for both WEs were recorded until they approached zero. The total amount of coulomb involved for the anodic or cathodic electron transfer was calculated according to equation 2.

\[
\text{Total Coulomb} = \int_0^t Idt \quad \text{.......................... (2)}
\]

Where \( I \) = anodic or cathodic current recorded at both WEs (C/s) and \( t \) = time period of the current measurement (s)

Finally, the theoretical coulomb present in the sample volume was compared with that measured from charging (cathodic) and discharging (anodic) currents.
2.3. Results and Discussion

2.3.1. Standard cyclic voltammetry of hexacyanoferrate

Prior to the testing of the described Twin-WE system, the use of the ITO electrodes for oxidizing and reducing common electron mediators was evaluated using conventional CV technique. Different concentrations of HCF (7.8 - 1000 μM) were tested (Figure 2.2). As expected, the typical electrochemical behavior of HCF was obtained with distinct oxidation and reduction peaks at approximately +0.322V and -0.058V, respectively. These peaks were reversible as reported in other similar aqueous systems (Garabagiu & Mihailescu, 2011). Also, the cyclic voltammograms obtained with different HCF concentrations were distinctive. This result confirms that the Twin-WE system could be effectively used to perform conventional CV for mediator detection.

![Cyclic voltammogram using ITO electrodes of different concentrations of HCF at a scan rate of 0.02 V sec⁻¹ between +0.7 V and -0.7 V.](image)

**Figure 2.2.** Cyclic voltammogram using ITO electrodes of different concentrations of HCF at a scan rate of 0.02 V sec⁻¹ between +0.7 V and -0.7 V.
2.3.2. A novel technique for mediator detection with two juxta-positioned working electrodes

In comparison to the conventional CV method using single-WE, the Twin-WE system consisted of two independently controllable juxta-positioned electrodes (distance of about 250 μm). Instead of rapidly sweeping the potential of the single WE to oxidize or reduce the mediators within the diffusion layer (boundary layer) of the electrode, a constantly oxidizing electrode faces a reducing counterpart would result in two opposing currents, which could be recorded simultaneously. Moreover, instead of a bulk electrolyte solution, the diffusion layers of both electrodes overlap. The presence of a mediator between the two WEs would cause an anodic and cathodic current respectively at the same intensity, with the mediator enabling electron transport via the diffusion of reduced mediator from the more reduced electrode to the more oxidized one.

2.3.2.1. The Twin-WE system could effectively detect hexacyanoferrate

Poising the juxta-positioned twin WEs, one at an oxidizing potential (e.g. +0.4V) and the other at a reducing potential (e.g. -0.4V) (i.e. “voltage window” of 0.8V across the HCF’s MP of +220 mV) enabled a continuous current generation at both WEs (Table 2.1). The currents were of similar values but with opposing directions and only observed in the presence of electron mediators. This result suggests that oxidation and reduction of the mediator (HCF) occurred simultaneously at a similar rate at the two WEs.
Table 2.1. Current intensity generated by poising the twin working electrodes at different potentials in the presence of 1mM HCF

<table>
<thead>
<tr>
<th>WE1 potential (V)</th>
<th>WE2 potential (V)</th>
<th>Current in WE1 (mA)</th>
<th>Current in WE2 (mA)</th>
<th>Current difference (mA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+0.4</td>
<td>-0.4</td>
<td>+0.255</td>
<td>-0.2596</td>
<td>0.5146</td>
</tr>
<tr>
<td>+0.3</td>
<td>+0.1</td>
<td>+0.1316</td>
<td>-0.1347</td>
<td>0.2663</td>
</tr>
<tr>
<td>+0.5</td>
<td>+0.3</td>
<td>+0.0011</td>
<td>-0.0012</td>
<td>0.0023</td>
</tr>
<tr>
<td>+0.1</td>
<td>-0.1</td>
<td>+0.0162</td>
<td>-0.0239</td>
<td>0.0401</td>
</tr>
</tbody>
</table>

A more narrow “voltage window” of 0.2 V was chosen by poising the two WEs at +0.1 and +0.3 V, respectively. These two potentials were respectively well below and beyond the MP of HCF and also resulted in a current each of which was similar but opposite to the other (Table 2.1). As expected, poising the WE potentials both at above (e.g. +0.5 V and +0.3 V) or below (e.g. 0.1 V and −0.1 V) the MP of HCF resulted in much lower current differences. This result affirms the underlying principle of the proposed mediator detection method with the Twin-WE system.

2.3.2.2. Signal improvement for mediator detection by optimizing the “voltage window”

Positioning a “voltage window” of the two WEs close to the mediator's MP resulted in a current difference, which was maintained by the oxidative and reductive half reactions of the two working electrodes. By recording the current difference between the twin WEs at different window positions, distinct peaks (of current difference) were obtained that revealed the MP of HCF of +0.22 V (Figure 2.3). Large voltage windows of 0.15–0.20 V gave relatively high current peaks of up to 0.1 mA (current density of 7 μA/cm²), whereas smaller voltage windows showed narrower peaks. This result clearly highlights
Chapter 2. New method for characterizing electron mediators using a thin-layer twin-working electrode cell

the potential of using the Twin-WE system for detecting redox active compounds in aqueous samples.

![Graph showing current differences measured between the facing twin gold WE in a solution of 1 mM hexacyanoferrate for different “voltage windows” (difference between the oxidizing and the reducing electrode given in V) used. The potential given as the X-axis represents the middle of the voltage window. For example, at a voltage window of 0.1 V and a given potential of +0.2 V, the reducing and oxidizing electrodes are at +0.15 V and +0.25 V, respectively. Based on the above optimization trials with various voltage window widths, a “voltage window” of 0.05 V was selected for subsequent tests. With the selected voltage window of 0.05 V, the sensitivity of using Twin-WE system for HCF detection was explored. As above, the current difference between juxta-positioned WE is recorded and plotted against the corresponding mid-point of the voltage window (i.e. average of the twin WE potentials) (Figure 2.4.A). The result indicated that with concentrations of less than 8 µM, the redox activity of HCF could still be detected, and a linear relationship between the current differences and the concentration of HCF was obtained (Figure 2.4.B). The peaks of the current difference also reveal the MP potential of the target mediator (HCF) at 0.225 V (Figure 2.4.A).
Chapter 2. New method for characterizing electron mediators using a thin-layer twin-working electrode cell

Figure 2.4. (A) Current difference peaks generated for various HCF concentrations by the ITO Twin-WE system. The “voltage window” between the twin WE was 0.05 V. (B) Relationship between the current difference peak between the twin WEs with a voltage window of 0.05 V and the corresponding HCF concentration.

2.3.3. Using the Twin-WE system as coulomb counter for mediator quantification
The defined electrolyte volume between the Twin-WE system could theoretically be used for sequential oxidation/reduction tests to determine the electron storage capacity of the mediators present. For these tests, both WEs were initially poised at the same reducing potential of +0.062 V (i.e. 0.150 V lower than the MP of HCF as determined in this study) to charge up (reduce) the mediators (HCF). This generated a cathodic current until the mediator was completely reduced. Subsequently an anodic current was obtained upon oxidation of the HCF (at +0.36 V, i.e. 0.150 V higher than the MP of HCF as determined in this study) in the confined volume between the two WEs. By integrating the current over the time interval, the total coulombs could be counted for the anodic and cathodic currents (Figure 2.5). The result indicated that the coulombs derived from both anodic and cathodic currents were well in line with the theoretically expected coulombs, indicating reliable electron balances for the system.
2.3.4. Comparison of Riboflavin monitoring by CV and the Twin-WE system

After analyzing a readily reactive chemical mediator (HCF), the electrochemical properties of RF, a well-known biologically important mediator was evaluated. CV for three different concentrations of RF was performed within the agar sealed WE chamber with only one functional WE (Figure 2.6). Detection of oxidation and reduction peaks of RF was possible only at concentrations ≥0.1 mM, as below which no clear oxidation and reduction peaks could be identified.
Figure 2.6. Cyclic voltammogram for 0.2 (blue), 0.1 (black) and 0.05mM (red) of Riboflavin with ITO electrode at a slow scan rate (1 mV/sec) between 0V to -0.95V. Higher scan rates of 20 mV per second failed to show peaks for RF under the current experimental conditions.

However, with the same electrodes and RF concentrations the Twin-WE system could generate distinctive peak signals at a much lower RF concentration of 0.05 mM (Figure 2.7). The peak was clearly identifiable at a potential of approximately -0.5 V (Figure 2.7). A linear correlation between RF concentrations and peak current differences between the two WE's was established (inset of Figure 2.7), suggesting that the Twin-WE system was more sensitive for quantification and more precise for midpoint potential identification than the traditional CV technique.
Chapter 2. New method for characterizing electron mediators using a thin-layer twin-working electrode cell

Figure 2.7. Current difference peaks generated for 0.2 (blue), 0.1 (black) and 0.05 mM (red) RF concentrations by Twin-WE system. Electrolyte solution within the WE chamber was separated from the counter electrode bulk solution by means of agar (see methods). Inset: Relationship of peak current with the corresponding RF concentration.

2.3.5. Multiple mediator detection in an active microbial culture
The above experiments confirmed that the Twin-WE system could effectively characterize and quantify both HCF and RF in a single-mediator containing system. Since microbial systems would typically contain more than one type of mediator, it was meaningful to test our system with a mixture of mediators. Hence, experiments were conducted to test whether our proposed method was effective for a real microbial system containing multiple mediators (Figure 2.8). The tested system was a mixture of RF (0.1 mM), HCF (0.03 mM) and an active Pseudomonas aeruginosa (WACC 91) broth culture. The Twin-WE system was operated as described above to establish the profile of current difference over a wide range of potentials (between −0.8 V and +0.8 V). The profile clearly revealed four distinct peaks at potentials of approximately −0.5, −0.35, −0.15 and +0.2 V (Figure 2.8.A). Based on the previous single-mediator experiments, the peaks at −0.5 V and +0.2 V were generated by RF and HCF, respectively. Additional peaks
occurred at −0.35 V and −0.15 V were most likely indicative of the mediators excreted by the *Pseudomonas aeruginosa* culture. In comparison, the use of CV with only one functional WE under the same conditions generated multiple oxidation and reduction peaks that were less distinctive (Figure 2.8.B). Therefore, our new method with the Twin-WE system offers a better qualitative and quantitative evaluation than conventional CV method.

**Figure 2.8.** (A) Current difference peaks obtained with the Twin-WE system for the mixture of RF (0.1 mM), HCF (0.03 mM) and *Pseudomonas aeruginosa* culture (50% v/v) grown in PMM (red line). Current differences between the two WE were generated by maintaining a voltage difference (“voltage window”) of 0.05 V between the twin electrodes. (B) Cyclic voltammogram for the same mixture with ITO electrode (scan rate 1 mV sec⁻¹) between +0.85 V to −0.95 V within Twin-WE chamber with only one functional WE. In both cases, control (blue line) was performed with PBS and PMM mixture (50:50).

### 2.3.6. Implication of the findings

Bacterial biofilms are significant ecosystems in which strong redox gradients are known to exist and where soluble electron mediators are known to mediate electron transfer within the biofilm. This has been reported in microbial fuel cells, bio-electrochemical systems (Smith et al., 2015), biosensors (Chaubey & Malhotra, 2002) and microbially induced corrosion (Zhang et al., 2015). The described Twin-WE system allows the
analysis of electrochemically active species in very small volumes. In comparison with CV using the same equipment the described scanning twin WE method enables lower detection limit and a distinctly higher resolution in the analysis of electron mediator mixtures.

Traditional electrochemistry methods rely on defined mass transfer conditions such as rotational speeds with rotating disc electrodes to establish reproducible boundary layer conditions (Bard & Faulkner, 2001). In contrast, the twin WE system described here consisted only of boundary layers that overlap between the two WEs, hence enabling a complete electron recovery in sequential oxidation/reduction tests (Figure 2.5). The use of such overlapping boundary layer leads to a controllable redox gradient between the two electrodes that can mimic redox gradients encountered in nature such as in biofilms, water sediment interfaces, and the human intestine, and may offer novel approaches for controlling and studying such biofilms. Further studies to exploit the use of this approach for studying microbial electron transfer in natural or engineered systems are warranted.

2.4. Conclusions
This study highlighted the suitability and potential of using the Twin-WE system for detecting and quantifying multiple electron mediators in a micro-scale microbial system. Unlike the conventional CV technique, the Twin-WE system adopted a unique method of maintaining a defined potential gradient (i.e. voltage window) across a thin-layer over a range of potentials. This approach enabled the generation of steady-state current signals, facilitating a more precise determination of the mid-potentials and concentrations of two model compounds (HCF and RF) within the thin-layer system. The method was also effective for detecting soluble mediators excreted by an active Pseudomonas aeruginosa culture. Overall, this unique method of manipulating redox
gradients across a thin-layer system could be further exploited to study extracellular electron transfer in important biofilm systems.
Chapter 3: *Pseudomonas aeruginosa* uses pyocyanin to extract energy from living cells¹

Abstract

Pyocyanin (PYO) acts as an electron mediator in planktonic cells and biofilm of *Pseudomonas aeruginosa* (PA). PYO is believed to cause host cell destruction while enabling PA to access the oxidation power of oxygen by relying on the spontaneous oxidation of reduced PYO (RedPYO) by oxygen. Here, we show that the RedPYO does not spontaneously react with oxygen unless catalyzed by active cells. Results presented support the view that PYO can harness electrons from target cells by oxidizing their NADH and subsequently, serves as electron donor for oxygen or nitrate respiring PA. This PYO mediated electron flow resembles syntrophic interspecies electron transfer, however here, it represents a novel form of energy parasitism. The discovery of this parasitic life style puts a new perspective on the role of PYO in biofilms, its natural soil environment and host infections.

¹This chapter has been submitted in *The ISME journal*
3.1. Introduction
Pyocyanin (PYO), chemically known as 1-hydroxy-N-methylphenazine, is a redox active compound secreted by the opportunistic bacterium *Pseudomonas aeruginosa* (PA). PYO is a well-known virulence factor and reported to play a major role in human infections, particularly during PA infected wounds (Sismaet et al., 2016), and in the lung of patients suffering from cystic fibrosis (CF) (Høiby et al., 2010; Palmer et al., 2007; Rada & Leto, 2013). The reversible redox behaviour of PYO involves the transfer of two electrons resulting in the interconversion between its oxidized (blue-green; OxPYO) and reduced (colourless; RedPYO) form (Bellin et al., 2014). The cytotoxic effect of PA on both prokaryotic (Hassan & Fridovich, 1980) and eukaryotic cells (Muller, 2002) has been linked to “PYO-induced oxidative stress”. The low molecular weight (210 Da) and zwitterionic property of PYO allow it to easily penetrate into the host cell where it can oxidize NADH (Britigan et al., 1992). The now reduced PYO is believed to be re-oxidized by molecular oxygen leading to the generation of superoxides (O$_2^-$) and peroxides (H$_2$O$_2$), which alter the redox homeostasis (oxidative stress) inside the host cell causing cell damage (Gardner, 1996; Gloyne et al., 2011; Muller, 2006; Ran et al., 2003) (Figure 3.1). Recently, PYO was found to be toxic to eukaryotic cells also under anaerobic condition (Barakat et al., 2014) suggesting oxidative stress is not the only cause for PA associated host cell destruction.
Chapter 3. *Pseudomonas aeruginosa* uses pyocyanin to extract energy from living cells

**Figure 3.1.** Overview of current understanding on possible role of pyocyanin in *Pseudomonas aeruginosa* interaction with host cells. Grey arrows indicate the direction of electron flow from NADH under OxPYO free condition.

The benefit for PA to use PYO for electron shuttling has been described in the literature. It is of general belief that the role of PYO is to mediate electron transfer from PA to molecular oxygen (Dietrich et al., 2008; Koley et al., 2011; Price-Whelan et al., 2007; Rada & Leto, 2013). For instance, the formation of a thin greenish layer (indicative of OxPYO) at the air liquid interface of a PA culture was interpreted as abiotic oxidation of RedPYO with oxygen (Price-Whelan et al., 2007). However, from bioenergetics (adenosine tri phosphate (ATP) generation) perspective, such diverted electron flow from NADH to OxPYO (instead of NADH to complex I of the electron transport chain) would uncouple oxidative phosphorylation and minimize ATP production of PA. Hence, it remains questionable how PA would benefit from this PYO-mediated electron transfer
mechanism to oxygen. From a bioenergetics perspective, it is also possible, and indeed, more sensible for PA to use the electron shuttling ability of PYO to harness reducing power from other living cells for their energy gain.

Recent observations in our laboratory showed that yeast cells exposed to OxPYO not only reduced the OxPYO but also released the resulting RedPYO that became available as a reducing power for PYO oxidizing PA cells. The aim of the current study is to evaluate whether PA can use PYO as an “electron extracting agent” to acquire reducing power from other cells.

3.2. Materials and Methods

3.2.1. Cultivation of *Pseudomonas aeruginosa*, PYO production and quantitation

*Pseudomonas aeruginosa* (WACC 91) was aerobically cultivated (in 500 mL baffled conical flask) for 4 days in 50% Luria-Bertani (LB) broth at 35°C under shaking condition (150 rpm) for maximum pyocyanin (PYO) production (indicated by intense blue green colour). PYO in the culture was microbially reduced by transferring this 4 days old culture into a 100-mL sealed serum bottle (flushed with nitrogen gas) and incubated at 35°C for 24 hours. Thereafter, the blue / green colour (indicative of the oxidized form of PYO), had vanished and the sterile filtrate served as a source of RedPYO. OxPYO supernatants were obtained by centrifuging (4600 rpm for 5 minutes) a 4 days-old PA culture followed by sterile filtration. Pure oxidized pyocyanin (Sigma-Aldrich) stock solution was prepared in acetone and diluted with deionized water to obtain different PYO concentrations. The concentration of OxPYO from PA culture was determined according to Lambert–Beer’s law, \( A = \varepsilon \times L \times C \) (Filloux & Ramos, 2014); where, \( A \) = absorbance at 690 nm, \( \varepsilon \) = extinction co-efficient of PYO (4130 M\(^{-1}\) centimeter\(^{-1}\)) (Dietrich et al., 2006), \( L \) = optical path-length of the cuvette (centimeter)
and C = concentration of PYO (M). Absorbance spectrum of PYO containing sample was recorded at wavelengths between 200 nm and 1000 nm using a scanning spectrophotometer (HP/Agilent 8453 UV-Vis) with 1 cm path length. Anaerobic sterile transfers of solutions were carried out by using nitrogen flushed 1mL syringe fitted with 0.22 µm Millipore syringe filter and 0.60 mm × 32 mm needle.

3.2.2. Reduced PYO as the electron donor for oxygen respiring PA
Two aliquots of 5 mL RedPYO containing PA culture (prepared as described above) were anaerobically transferred from the sealed vial to a degassed (by purging nitrogen gas) test tube fitted with rubber stopper. One of the samples was anaerobically sterile filtered to allow comparison of full culture and cell free filtrate. Both test tubes were then exposed to air with rapidly shaking for 1 minute to oxygenate the liquid mixture. Oxidation of PYO was compared by recording the absorbance spectrum of both samples (PA cell containing culture was filtered prior to the spectrophotometric scanning) before and after oxygenation. A similar experiment was also performed using 100 µM pure pyocyanin (Sigma Aldrich) in 5 mL of 100 mM phosphate buffer, (PBS; made from KH$_2$PO$_4$ and Na$_2$HPO$_4$ to pH 7.3): 0.1 mL of washed PA cell pellet was added to two 5 mL pure OxPYO containing oxygen free (N$_2$ degassed) degassed vials to generate RedPYO. After the reduction of OxPYO (indicated by change in colour from blue to colourless), PA cells from one of the vials was filtered out anaerobically and both vials were exposed to air for 1 minute. Thereafter, the absorbance spectrum for both samples was obtained by following the above-mentioned technique.
3.2.3. Reduced PYO as the electron donor for nitrate respiring PA

Since PA is a known denitrifier, nitrate could be tested as an alternative electron acceptor for RedPYO oxidation. A PA culture was anaerobically grown for 48 hours in a LB medium supplemented with sodium nitrate (NaNO₃; 10 mM). Thereafter, the culture was centrifuged and the cell pellet was washed twice with sterile 100 mM PBS. The washed PA cell pellet (0.1 mL) was then injected into two degassed test tubes containing 5 mL RedPYO leading to the final optical density (OD₆₀₀) of 0.75. A 0.1 mL of N₂ purged NaNO₃ solution (0.1M) was injected into one test tube, while the second test tube was spiked with 0.1 mL N₂ purged PBS (0.1M) to serve as a control. Changes in absorbance at 690 nm was monitored for 30 minutes for both test tubes and recorded. Subsequently, the cell suspensions were anaerobically filtered and the absorbance spectrum of the filtrate recorded.

3.2.4. Electrochemical setup and general operation

A thin electrochemical cell with a modified (2 mm thick) non-conductive perspex frame sandwiched between two adjacent indium tin oxide (ITO) coated glass slides (working surface area 13.5 cm²) was used to perform electrochemical experiments as previously described (Hassan et al., 2017). The cell was operated as a mini-bioreactor, with an inlet, outlet and ion exchange channels machined into the non-conductive frame (Figure 3.2). Two capillary tubings (Cole Parmer P/N 6417-21 24TW) were connected to the inlet and outlet channels for sample loading and discharge, respectively while the two oppositely drilled holes (1.5 cm up from the bottom of the frame) and all the edges of the WE chamber were sealed with 1.5% molten agar gel to enable ionic migration between working and counter electrode (CE). The perspex frame and ITO electrodes were mounted together (using adhesive tape) to construct the WE chamber (working volume of 1.3 mL). A graphite rod (4 mm in diameter and 105 mm in length) and a silver-silver
chloride (Ag/AgCl) electrode in saturated KCl (MF-2052, BASi, USA) was used as the CE and reference electrode (RE), respectively (Figure 3.2).

Figure 3.2. Schematic of the electrochemical setup used in this study.

The sample tested was injected to the WE chamber by syringe through the inlet port and the chamber was inserted into 50 mL of PBS (100mM) as electrolyte where also the CE and RE were positioned (Figure 3.2). All electrodes were connected to a custom-made potentiostat (built by Murdoch University electronics workshop) as described in our earlier work. All the potentials mentioned in this article are referred against Ag/AgCl reference electrode (+0.210 V vs the standard hydrogen electrode, SHE). All electrochemical experiments were controlled and monitored by an automated
LabVIEW™ software program interfaced with a computer through a data acquisition card (LabJack U12™).

### 3.2.5. Electrochemical evaluation for PYO mediated electron extraction

The ability of PYO to extract electrons from host cells was investigated electrochemically using yeast (*Saccharomyces cerevisiae*) and *E. coli* as the surrogate of eukaryotic and prokaryotic cells, respectively. The effect of OxPYO on PA was also evaluated. The yeast was aerobically grown on yeast extract peptone dextrose (YPD) medium containing (L⁻¹ in deionized water): D-glucose (20g), peptone (10g) and yeast extract (10g) at 27°C for 48 hours. *E. coli* and PA were grown aerobically on LB medium at 35°C for 24 hours. The OD of the cultures were measured at 600 nm before centrifugation (at 4600 rpm for 5 min) and the supernatant was discarded to obtain cell pellet. Cells were washed twice with sterile 100 mM PBS before being injected into the WE chamber.

The sterile filtrate of an aerobically grown PA culture with approximately 100 µM OxPYO was loaded into the WE chamber through the inlet tube and the chamber was vertically placed in an electrolyte containing vessel (Figure 3.2). Thereafter, both WEs were poised at -150 mV until all OxPYO was fully oxidized (as indicated by steady zero anodic current). After 30 minutes, 0.1 mL of active yeast cell pellet was injected into the WE chamber (final OD₆₀₀ value of 4.64) and the current generated at WE1 (at -150 mV) was recorded while WE2 was disconnected (inactive). After approximately 14 h from the addition of yeast cells, D-glucose was added from a stock solution (100mM) to reach about 7 mM to the WE chamber to replenish the reducing power (NADH) inside the yeast cell. A control experiment was done by following the same procedure with 100mM PBS instead of OxPYO.
3.2.6. Spectrophotometric evaluation for PYO mediated electron extraction
A 0.05 mL washed yeast cell pellet was injected into a test tube containing 5 mL OxPYO (approximately 110 µM) solution (absorbance spectrum was pre-recorded) leading to the final OD₆₀₀ value of 0.71. Immediately, the oxygen in the headspace was removed by purging with nitrogen and the opening was sealed with a rubber bung. Changes in absorbance at 690 nm was recorded over time and finally, the absorbance spectrum for OxPYO of the anaerobic filtrate was recorded.

3.2.7. PYO mediated electron extraction in PA-Clostridium mixture
*Pseudomonas aeruginosa* was anaerobically grown in 50% diluted LB broth containing 10 mM NaNO₃ at 35°C for 48 hours whereas *Clostridium pasteurianum* was cultivated anaerobically in tryptic soy broth (TSB) at 37°C for 48 hours. Both cultures were centrifuged (at 4600 rpm for 10 mins) and cell pellets were washed twice with 50 mM PBS (N₂ purged PBS for washing *C. pasteurianum*) to remove residual organic substrate. 0.1 mL of washed PA cell pellet was inserted by syringe into 2 mL of 50mM PBS (leading to the final OD₆₀₀ of 3.1) within a sealed reaction vessel (5 mL) to which an optical DO probe (Vernier™ software & Technology) was pre-fitted 0.5 mm above the bottom of the vessel. The oxygen uptake rate (OUR) of the washed PA cells was monitored and then continuous air supply was maintained for about 3 h until the endogenous respiration rate decreased to less than 10 mg/L/h. A twice washed 0.1 mL *C. pasteurianum* cell pellet was injected into the same reaction vessel (leading to OD₆₀₀ of 3.4) and was gently agitated. The effect of *C. pasteurianum* cell addition on the OUR of PA was recorded for 10 minutes. Thereafter, 200 µM pure OxPYO (Sigma Aldrich) was added to the same mixture and the change in OUR monitored.
3.2.8. Continuous oxidation of RedPYO by PA with oxygen
The ability of PA to aerobically oxidize RedPYO was tested electrochemically using the electrochemical set-up described above (Figure 3.2). To reduce PYO (150 µM final concentration, added from pure chemical (Sigma-Aldrich CN: P0046), the ITO working electrode was poised at -600 mV. At this potential, the presence of OxPYO generates a cathodic current. The addition to the WE chamber of OxPYO caused such a current until it was completely reduced. 0.1 mL double washed PA cell pellet (grown aerobically) was injected into the WE chamber (leading to OD₆₀₀ value of 2.3). After 15 min and the establishment of a stable, low background current, air was purged to the WE chamber (through the inlet tube) for 1 minute and the current generated due to oxidation of the RedPYO by PA was recorded. A control experiment was also performed by following the same procedure using 100 mM PBS instead of OxPYO.

3.2.9. Effect of PYO on oxygen uptake rate of yeast
Two mL of 100 µM pure OxPYO in 50 mM PBS was added to a small (5mL) cylindrical glass reaction vessel of which the opening was sealed with an O-ring and the oxygen probe in such a way that no gas phase or even air bubble was present inside the reaction vessel and gas exchange with the atmosphere was eliminated. Dissolved oxygen (DO) was monitored using an optical DO probe (Vernier™ software & Technology) interfaced to the computer through a Vernier LabQuest® Mini. Thereafter, 0.1 mL of double washed (with 50 mM PBS) yeast cell pellet was added to the same beaker (final cell concentration OD of 1.4) and the changes in DO was recorded over time to derive the OUR. Similar experiments were performed with the same amount of yeast cell pellet in 50 mM PBS without OxPYO as the control experiment.
3.3. Results

3.3.1. Abiotic versus biotic oxidation of RedPYO

It is well known and described both by experiments (Wang & Newman, 2008) and observations (Price-Whelan et al., 2007) that RedPYO can readily react with oxygen to become oxidized. Typically, these experiments refer to the very apparent colour change from near-colourless or faint yellow to blue-green when exposing oxygen depleted PA cultures to air. To investigate the potential contribution of PA cells to the RedPYO oxidation by oxygen, oxidation of RedPYO (for both PYO obtained from PA culture and commercial pure PYO) was recorded both in the presence and absence of PA cells (Figure 3.3). As described in the literature, exposure of the PA containing RedPYO to oxygen resulted in immediate colour change and hence, oxidation of PYO within 30 seconds (Figure 3.3.A and 3.3.C).

Investigation of the absorbance spectrum (Figure 3.3.A and 3.3.C) showed the typical evidence of oxidized PYO only in the biological sample but not in the cell free solutions. Further, the difference between the two spectrums revealed a clear net absorbance change at 690 nm (Inset of figure 3.3.A and 3.3.C), which is the wavelength of PYO absorbance ($\lambda_{\text{max}}$). Results also suggested that the oxidation of RedPYO by oxygen was dependent on the presence of PA rather than a simple chemical reaction. The RedPYO in the absence of PA did not show signs of reaction with oxygen over more than 30 minutes (Figure 3.3.B and 3.3.D). Even the exposure to air oxygen for several days did not result in measurable PYO oxidation (data not shown).
Chapter 3. Pseudomonas aeruginosa uses pyocyanin to extract energy from living cells

Figure 3.3. PA catalyzed oxidation of RedPYO in whole PA culture (A and B) and pure 100 µM PYO solution (C and D), respectively. A/C. Spectrophotometric spectrum of RedPYO after exposure to air (oxygen) for 30 seconds in the presence (green line) and absence (brown line) of Pseudomonas aeruginosa. Inset: difference of absorbance between the biotic and abiotic absorbance spectrum B/D. Spectrophotometric spectrum of RedPYO during exposure to air oxygen for 0 (solid line), 15 (dashed line) and 30 (dotted line) minutes. Inset: Absorbance ratio (690nm/500nm) spectrum of PYO. All experiments were performed in triplicates.

The inability of oxygen to oxidize RedPYO in the absence of PA was confirmed as a reproducible phenomenon (data not shown). Sterile filtration of RedPYO containing PA cultures always rendered the filtrate inert with respect to oxidation by oxygen, while non filter-sterilized samples were readily oxidized when supplied with oxygen. The same conclusion was deduced from the results obtained with commercial PYO (Sigma
Aldrich), which remained reduced (colourless) upon exposure to oxygen even for an extended period (>one month).

The finding that air oxidation of RedPYO required the presence of live PA cells suggests that perhaps PA might actively oxidize PYO using oxygen as terminal electron acceptor. If this is the case, then PA may also oxidize PYO with terminal oxidants other than oxygen. To test this hypothesis, oxidation of RedPYO by active PA cells was tested in the presence of nitrate as the terminal electron acceptor. From the result, it is evident that the nitrate containing PA culture could also oxidize RedPYO, while, as expected, in the absence of nitrate, RedPYO stayed reduced (Figure 3.4.A). This result demonstrates the capability of PA to utilize RedPYO as electron donor for respiration under anoxic conditions using nitrate as the terminal electron acceptor.

Figure 3.4. PYO oxidation by PA and reduction by yeast cells A. Spectrophotometric spectrum of RedPYO in the presence of *Pseudomonas aeruginosa* (NO$_3$ reducing culture) for 30 minutes, in the presence (green line) and absence (brown line, 10 mM PBS added as control) of 10 mM NO$_3$. PA cell pellets (0.1 mL) were added to two degassed test tubes each containing 5 mL filtered RedPYO. Changes in absorbance in both test tubes were recorded over 30 minutes and finally, samples were withdrawn, filtered and scanned for absorbance spectrum. Inset: Changes in absorbance over time. B. Spectrophotometric spectrum of OxPYO (approximately 110 µM) before (green solid line) and after (dotted brown line) exposure to yeast cell for 60 min.
under anaerobic condition. **Inset:** Changes in absorbance over time. All experiments were performed in triplicates.

### 3.3.2. OxPYO had an electron draining effect on live cells while PA was unaffected

The above findings, so far suggested that RedPYO could serve as electron donor to PA under both oxic and anoxic condition. Accordingly, reactions that can reduce OxPYO to RedPYO may potentially serve as electron source for the RedPYO oxidizing PA biofilm. As it is well-known phenomenon that NADH serves as electron donor for oxidized pyocyanin (Britigan et al., 1992), it can be deduced that the electron source for PYO reduction is the NADH of other cells. A batch test using starved and washed yeast cells showed that under anoxic conditions, extracellular OxPYO becomes entirely reduced (Figure 3.4.B). This shows that OxPYO has the capacity to act as an oxidant to extract electrons from live cells into the surrounding bulk solution in the form of RedPYO.

To test whether PYO can act as a mediator that can repeatedly extract electrons from live cells as long as it is being continuously oxidized, electrochemical experiments were performed. OxPYO (100 µM) was added to the WE chamber of the electrochemical cell and the anodic current was monitored (Figure 3.5.A). The addition of yeast cells (0.1 mL pellet at approximately 1.5 h) caused a 10-fold increase in current while no current change was observed in the control (yeast cells without OxPYO). This increase in current was attributed to the reoxidation of the RedPYO. The current was somewhat diminished over the next 14 hours, likely due to the exhaustion of electrons (i.e. NADH) available from the yeast cells. To confirm whether this was the case, the yeast cells were supplemented with glucose (approximately 7 mM). Clearly, the addition of glucose resulted in profound and repeatable current peaks of around 24 µA while no response was recorded in the control without PYO (Figure 3.5.A). This suggests electron flow from glucose to OxPYO was catalyzed by the yeast cells. From the fact that glucose
oxidation leads to the reduction of NAD$^+$ to NADH and that NADH reduces OxPYO (Britigan et al., 1992), an overall electron flow from the sugar to the ITO anode via NADH and PYO can be inferred (i.e. glucose → yeast → NADH → PYO → electrode → current).

Figure 3.5. Electrochemical monitoring of PYO mediated electron extraction from living cells A. Anodic current signal generated after the addition of yeast cells and subsequent glucose (7 mM) spikes inside the OxPYO (100 µM) containing electrochemical cell WE chamber (blue line) where the WE was poised at -150 mV (oxidizing potential for PYO). Red line indicates the current signal for the same test in PBS (100 mM, pH 7.3). OxPYO and PBS was loaded into the WE chamber without headspace to avoid O$_2$ entry. B. Anodic current signal generated after the insertion of washed *E. coli* (blue line) and *Pseudomonas aeruginosa* (black line) inside the OxPYO (100 µM) containing Twin-WE chamber.

The effect of OxPYO on prokaryotic cells was explored electrochemically using *E. coli* and *Pseudomonas aeruginosa*. The same amount of starved and washed bacterial cell pellet (48 hours old) were separately injected to the electrochemical cell chamber preloaded with OxPYO (100 µM), and the response of anodic current was monitored. An immediate rise in anodic current was recorded only with the addition of *E. coli* but
not PA (Figure 3.5.B). Since the WE was not pre-colonized with any cells (i.e. plain electrode), the recorded current must be caused by the re-oxidation of RedPYO (produced in exposure to bacterial cells) at the electrode.

Results suggest that OxPYO was about 20 times more effective in draining electrons from washed *E. coli* cells than washed PA cells. The interesting finding that washed PA cells were not able to effectively reduce OxPYO is in line with general observations that under carbon starvation (e.g. end of growth) PA cultures have a blue green appearance even during prolonged exposure to oxygen free conditions. Whether PA has a mechanism that protects its cells (and its own NADH) from being oxidized by OxPYO is unclear.

The above evidence is confirmative of the reduction of OxPYO by yeast cells and the biological oxidation of RedPYO by PA rather than its chemical oxidation by oxygen. In combination, these observations logically lead to conclusion that PA can use PYO as a means to acquire electrons (reducing power) from other cells such as *E. coli* or yeast cells and use the electrons for oxygen or nitrate reduction. As the dissimilatory use of oxygen or nitrate in aerobic bacteria generally proceeds via electron transport phosphorylation, being a respiring bacterium, the oxidation of PYO by PA in the presence of oxygen and nitrate should lead to ATP production. This could be described as a parasitic interspecies electron transfer from donor cells (here, the *E. coli* or yeast cells) to PA.

OxPYO is known to become reduced by NADH of the live cells and here, we found PA can actively oxidize RedPYO in the presence of suitable electron acceptors. Hence, it is expected that PYO addition to a mixture of live PYO reducing cells and PA leads to simultaneous reduction and oxidation of PYO. To test whether such interspecies electron transfer could be mediated by PYO, cells of the strict anaerobe *Clostridium pasteurianum* were used as electron donor. Controls with washed cells of *C. pasteurianum* and PA (i.e. in the absence of PYO) showed low oxygen uptake rates (Inset of Figure 3.6.A) and the
addition of OxPYO to each had no significant effect on oxygen uptake. In contrast, the addition of PYO to the mixture of both cells increased the respiration rate, arguably of PA 2.5-fold (Figure 3.6.A). This suggests that PYO enabled PA to extract reducing equivalents from NADH of the *C. pasteurianum* cells. Whether such an inadvertent loss of reducing equivalents by the electron donating cell is detrimental or of potential benefit (e.g. dumping electrons to enable acetate excretion and benefiting from the acetyl-kinase reaction to produce ATP) to the target cells is discussed below.

**Figure 3.6.** Stimulation of microbial electron flow by pyocyanin. A. Stimulating effect of OxPYO addition on oxygen uptake rate (OUR) of starved PA cells in the presence of the anaerobe *Clostridium pasteurianum* (CP) as electron donor. **Inset:** Non-stimulating effect of PYO on individual pure cultures of PA and CP. B. Effect of oxygen addition (from 3 to 4 min) on cathodic current generation (caused by OxPYO accepting electrons from the ITO electrode) by washed PA cells in the presence (red spheres) and absence (black squares) of PYO. C. Oxygen uptake by washed yeast cell suspension (added to PBS buffer at 12 min) in the presence (red line) and absence (black line) of OxPYO (100 µM).
Chapter 3. *Pseudomonas aeruginosa* uses pyocyanin to extract energy from living cells

The ability of PYO to act as a continuous electron carrier from a reducing site to oxygen supplied PA cells was tested electrochemically using an ITO electrode as the reducing agent for PYO reduction. The addition of OxPYO to the ITO electrode poised at -600mV resulted in a cathodic current until the OxPYO was reduced (data not shown). In the presence of oxygen, PA could regenerate OxPYO resulting in renewed current (Figure 3.6.B), while the same washed cells of PA did not generate a current when PYO was not present. This result provides a further example of PA using PYO for accessing electrons for oxygen respiration from a suitable reducing process.

It could be expected from the above result with electrode as the PYO reducing agent that an electron transfer from yeast cells (instead of electrode) to PA would also occur in the presence of oxygen as the electron acceptor. However, after yeast cells had reduced OxPYO, the subsequent exposure of the yeast cells-PYO mixture to air re-oxidized the RedPYO, despite the absence of PA. This surprising effect of eukaryote catalyzed oxidation RedPYO by oxygen was reproducible and was also observed with soil fungal mycelium and human intestinal HCT-8 cells (data not shown). It implies that in the presence of respiring cells PYO oxidation and reduction could be simultaneous, as it is known for respiration un-couplers.

To test whether PYO could possibly have an uncoupling effect (by-passing electron transport from NADH to the electron transport chain and hence preventing ATP formation) on eukaryotic cells, OxPYO was added to starved yeast cells and its effect on oxygen consumption monitored (Figure 3.6.C). The addition of OxPYO increased the background endogenous respiration by about 65%, showing that PYO expedited the transfer of electrons from NADH to oxygen, presumably via by-passing the electron transport chain.
3.4. Discussion

Our results show that the well-studied electron mediator, pyocyanin (PYO) produced by PA does not react with oxygen unless catalyzed by living cells. This finding requires a revision of the generally portrayed role of PYO in PA infected wounds or cell tissue such as lung cells. From the results that OxPYO can extract electrons from other prokaryotic or eukaryotic cells (Figures 3.5 and 3.6.A) and make them available to PA as an electron donor for oxygen or nitrate respiration, a novel beneficial role of PYO for PA can be envisaged both in the natural soil environment and in opportunistic pathogenic biofilm formations.

The extraction of electrons from NADH of active cells typically represents a loss of energy for these cells if the NADH could otherwise be used for respiration. In non-respiring, fermenting microbes, the accumulation of NADH can represent a metabolic barrier disabling the uptake of substrate such as sugars or fatty acids. In such cases, the loss of electrons (re-oxidation of NADH) is beneficial to such cells as is known for obligate hydrogen producing acetogens that reduce protons to H₂ as an electron dumping mechanism termed interspecies electron transfer (Stams & Plugge, 2009). The possibility of PA extracting electrons and thereby generating a mutually beneficial syntrophic electron transfer seems possible. It is known that OxPYO has the capability to non-enzymatically oxidize NADH of the live cells, whereas in PA cells intracellular reduction of PYO has recently been reported to be catalyzed by pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes (Glasser et al., 2017). This implies that PA may be able to control NADH driven reduction of OxPYO whereas host cells cannot.

The existing view of PYO as an electron mediator between oxygen and PA would make sense in situations where PA is oxygen limited. Arguably, the exposure of PA biofilms to atmospheric oxygen in lung and wound infections raises skepticism as to why PA
would have to rely on PYO to mediate extracellular electron transfer (EET) to oxygen. It seems more obvious for obligate anaerobic bacteria such as Faecalibacterium to benefit from the use of exocellular mediators (e.g. flavin) to shuttle electrons to oxygen under oxygen-limiting environment (Khan et al., 2012). In the light of results shown, it makes sense that if PA has access to oxygen it could profit from PYO as a delivery mechanism of electrons extracted from host cells or other prokaryotic cells that may be present in biofilms. The same electron extraction process could also work in anoxic PA biofilms (e.g. in the lung of cystic fibrosis patient) where nitrate serves as the electron acceptor (Hassett et al., 2002).

PYO has been shown to contribute in increased H$_2$O$_2$ levels, particularly, in lung tissue of cystic fibrosis patients (Britigan et al., 1992). It is thought that H$_2$O$_2$ originates from direct chemical oxidation of RedPYO with oxygen (Gloyne et al., 2011). Our findings that this chemical reaction does not occur unless catalyzed by active cells requires an alternative explanation for the observed production of reactive oxygen species (ROS) such as superoxide (O$_2^-$) and its product peroxide (H$_2$O$_2$). By accepting that PYO does not chemically react with oxygen but requires the presence and presumably catalysis of respiring cells, it could be proposed that the observed formation of oxygen radicals is originated from the catalyzed oxidation of RedPYO. How the catalysis of electron transfer from RedPYO to O$_2$ is likely mediated is unclear, however the reduced form of phenazine methosulphate, a phenazine derivative like PYO has been shown to transfer electrons to mammalian cytochrome c (Bisschop et al., 1979). Given that cytochrome c accepts a single electron (Bowman & Bren, 2008) and RedPYO carries two electrons, the intermittent production of a radical species such as superoxide during the electron transfer from RedPYO to cytochrome c is understandable. Indeed, ROS are known to be by-products from regular respiration including mitochondrial respiration (Turrens,
2003). Whether this potential catalytic PYO oxidation via cytochrome c is in fact a reason for \( \text{H}_2\text{O}_2 \) accumulation in PA infected host tissue such as the lung, needs further study.

### 3.4.1. Proposed mechanism

Based on the findings of this study, we postulate a parasitic energy harvesting mechanism operated by *Pseudomonas aeruginosa* (Figure 3.7). OxPYO, after being produced by PA cell, diffuses into the host cell and oxidizes NADH. Oxidation of NADH by OxPYO would thus uncouple the usual direction of the electron flow via electron transport chain causing an obstruction in ATP production in the host cell. After being reduced by NADH, the RedPYO can diffuse out of the host cell to serve as electron donor for PA in the presence of oxygen or nitrate. The higher redox potential of the electron transport chain proteins (Ubiquinone -165 mV; Cytochrome b -133 mV and Cytochrome C1 +10 mV) (Nelson et al., 2008) than PYO (-244 mV) could thus enable a continuous supply of electrons to the ETC of PA for ATP generation. Nitrate dependent oxidation of RedPYO by PA could still enable electron transport phosphorylation by using complex I or II proteins (up-stream of cytochrome b) as the initial electron acceptors for RedPYO oxidation. Ultimately, such reversible redox reaction would drain electrons out of the host cell resulting in an exhaustion of reducing power and potentially leading to death of the host cell by uncoupling the electron flow responsible for energy (ATP) production.
Chapter 3. Pseudomonas aeruginosa uses pyocyanin to extract energy from living cells

Figure 3.7. Proposed mechanism of the PYO mediated “energy parasitism” operated by Pseudomonas aeruginosa with its potential host cells. The blue box inside the host cell shows the possible reactive oxygen species (ROS) generating reactions of RedPYO in the presence of oxygen. Grey arrows show undisturbed electron flow from NADH to the electron transport chain. For simplification cell organelles are not shown.

3.5. Conclusion

Overall, the results shown encourage to revisit the role of the high impact microbe P. aeruginosa in different environments such as the soil, biofilms and mammalian cell tissue. For example, preliminary experiments suggest that PYO produced by PA can extract electrons also from fungal mycelium and plant roots (data not shown). To what extent the newly revealed role of PYO can help in devising improved control actions against this opportunistic pathogen could be the subject of future research.
Chapter 4: Pyocyanin accelerates *Pseudomonas aeruginosa* associated aerobic corrosion

Abstract
Microbially influenced corrosion (MIC) is a complex bio-electrochemical process where the exposure of the metal to microorganisms and their metabolic products causes dissolution of metal ions. This study aims at evaluating the role of pyocyanin (PYO) in *Pseudomonas aeruginosa* (PA) associated aerobic corrosion of steel. Results showed that PYO can more efficiently harness electrons from the steel than oxygen alone at potentials less than -0.3 V (vs. Ag/AgCl). The reduced PYO thus generated (RedPYO) can subsequently be oxidized by oxygen. The corrosion rate determined by the release of dissolved iron from the steel was increased by two-fold in exposure to PYO compared to a PYO free electrolyte under oxygen saturated condition. This increase in corrosion rate can be interpreted due to the existence of a PYO mediated electron flow from the steel to the oxygen which accelerated the cathodic half corrosion reaction. PA cells can also be benefitted from this electron flow to generate cellular energy (ATP) using RedPYO as the electron donor for oxidative phosphorylation. To our knowledge, this is the first study to demonstrate the independent role of biological electron mediator in influencing aerobic corrosion rate by cathodic stimulation.
4.1. Introduction

Metal corrosion is an electrochemical process causing the deterioration of metals due to the release of electrons or ions from the metal (Kip & van Veen, 2014). The adverse effect of metal corrosion on industries and municipal services such as oil refinery, shipping, construction, sewage and drinking water supply systems etc. has become one of the major global challenges (Li et al., 2015b). Metal corrosion involves the presence of simultaneous anodic and cathodic half reactions on the metal surface where the electrons from the anodic site are transferred to the cathodic site and extracted by the electron acceptors such as oxygen (Hamilton, 1985). The loss of electrons from the cathodic site by the electron acceptor causes the dissolution of metal ions from the anodic site resulting in corrosion (Hamilton, 1985).

Anodic half reaction: \[ \text{M} \rightarrow \text{M}^{2+} + 2e^- \]

Cathodic half reaction: \[ \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} + 2e^- \rightarrow 2\text{OH}^- \]

The products formed from the above two half reactions react with each other to generate corrosion product which in case of iron is the following:

\[ \text{Fe}^{2+} + 2\text{OH}^- \rightarrow \text{Fe(OH)}_2 \] (Iverson, 1987)

Some metal corrosion processes are occurred due to the electrochemical reactions initiated and accelerated by microorganisms present on the metal surface and known as microbially influenced corrosion (MIC) (Cord-Ruwisch, 2000; Ford & Mitchell, 1990). MIC is one of the major types of corrosion causing 20% of all corrosion damages (AlAbbas et al., 2013) and the annual MIC related industrial loss in Australia was estimated to be AUD $6 billion (Javaherdashti & Singh, 2001). The major types of bacteria involved in MIC are sulfate reducing bacteria (SRB) (Beese-Vasbender et al., 2015), iron oxidizing bacteria (Liu et al., 2015) and sulfur-oxidizing bacteria (Lanneluc
et al., 2015). Microorganisms can influence the corrosion process in different ways such as cathodic hydrogen utilization to increase metal dissolution at the anode, degradation of the protective coatings of metal, degradation of the corrosion inhibiting chemicals and production of corrosive metabolites (Coetser & Cloete, 2005).

In MIC, the electron transfer from the metal to electron acceptors is often considered as rate limiting (Jia et al., 2017b) and based on the source of electrons for this cathodic half reaction, MIC can be categorized into two major types namely chemical MIC (CMIC) and electrical MIC (EMIC). In CMIC, the hydrogen layer formed on the metal surface serves as the electron donor for the microorganisms under anaerobic condition (Enning et al., 2012) whereas the microbes associated with EMIC can extract electrons directly from the metal surface (Venzlaff et al., 2013). Electroactive microorganisms operate the EMIC process by establishing an electron flow between the metal and their terminal electron acceptor such as sulfate ($\text{SO}_4^{2-}$) to thrive under such condition (Venzlaff et al., 2013; Xu & Gu, 2014). MIC has also been reported to be a result of synergistic interactions amongst microbial community members where cascade of biochemical reactions take place to utilize steel as the electron donor by SRB and carbon dioxide ($\text{CO}_2$) as the electron acceptor by acetogens (Usher et al., 2015).

Most of the MIC studies were performed with nitrate and sulfate reducing bacteria under anaerobic condition (Cord-Ruwisch & Widdel, 1986; Enning & Garrelfs, 2014) and the role of aerobic biofilms in influencing corrosion under aerobic condition remains controversial. Given that the formation of aerobic biofilms on stainless steel surface has been reported to enhance cathodic oxygen reduction in bioelectrochemical systems (BES) such as microbial fuel cell (MFC) (Bergel et al., 2005; Dumas et al., 2007) and that aerobic biofilms have also been reported to act against corrosion by forming an iron
oxide-phosphate layer on a mild steel surface (Chongdar et al., 2005), it is important to revisit the role of microorganisms in influencing steel corrosion under aerobic condition.

*Pseudomonas aeruginosa* (PA), a Gram-negative aerobic bacterium has been extensively used as a model organism in MIC studies. For instance, its role in different steel corrosion under anoxic condition where nitrate (NO$_3^-$) was provided as the electron acceptor (Jia et al., 2017a; Jia et al., 2017b; Jia et al., 2017c). Some studies showed that the presence of PA biofilm on a steel surface causes dissolution of high levels of Fe and Cr under aerobic condition (Li et al., 2017; Li et al., 2016).

The addition of electron mediators (FAD and riboflavin) in PA culture has already been shown to influence the anaerobic corrosion rate where these mediators were proposed to mediate an electron flow from the steel surface to PA (Jia et al., 2017b). In their study, the corrosion rate was increased by 27% and 14% with the addition of FAD and riboflavin, respectively to a carbon steel (C1018) exposed PA culture. Although these non-PA produced electron mediators didn’t cause significant increase in corrosion rate, this finding triggers the necessity of investigating the potential role of PA produced electron mediators in PA associated corrosion. PA has been reported to catalyze cathodic electron transfer from the electrode to both nitrate (Virdis et al., 2011) and oxygen (Cournet et al., 2010). PA catalyzed oxygen reduction process was proposed to be PYO independent and mentioned as the direct electron transfer from the cathode to PA cells (Cournet et al., 2010). However, the potential role of pyocyanin (PYO) in PA associated aerobic corrosion remains uninvestigated. Understanding the role of PYO in aerobic corrosion would lead to scheme the possible mechanism for PA biofilm driven aerobic corrosion.

In our previous study, we found that oxidized PYO (OxPYO) is capable of extracting electrons from both prokaryotic and eukaryotic living cells and the reduced form of PYO
(RedPYO) can be utilized as the electron donor by PA in the presence of nitrate or oxygen (Chapter 3). The RedPYO oxidizing capability of PA under both oxic and anoxic condition is indicative of the use of RedPYO as an electron donor for the respiration of PA cells. Since OxPYO has been found to be an electron extracting agent from living cells, it may also harness electrons from steels when the potential of the steel is less than the mid-potential of PYO (−244 mV vs. Ag/AgCl). In a PA biofilm exposed steel, the ability of OxPYO to accept electrons from the steel and the subsequent oxidation of RedPYO by PA in the presence of oxygen will generate a continuous electron flow from the steel to oxygen by PYO and PA. Since the establishment of such a PA mediated electron flow would in principle accelerate the dissolution of metal in the anodic site, validation of this hypothesis is useful for designing strategies to prevent MIC under aerobic condition.

Specific research questions of this study are:

1. Can OxPYO also extract electrons from corroding steel?
2. Can PYO catalyze the electron transfer from corroding steel to oxygen?
3. Does the presence of PYO increase aerobic corrosion of steel?
Chapter 4. Pyocyanin accelerates Pseudomonas aeruginosa associated aerobic corrosion

4.2. Materials and methods

4.2.1. Coupon preparation, electrolyte and chemicals

Two different types of steels namely carbon steel (AISI 1030) and stainless steel (SS304) were used in this study. The compositional details of these steels are presented in Table 4.1. All the steel coupons were wet ground up to 600 grit finish, rinsed in acetone and dried with nitrogen prior to each experiment. Indium tin oxide (ITO) coated glass electrode (resistance 8–12 Ω/sq; RileySupplies, Richmond Hill, Canada) was used to develop a polarization curve in phosphate buffer saline (PBS;100 mM; pH 7.3). Bicarbonate buffer (BCB) solution was used as the electrolyte for the experiments conducted with carbon steel (CS) whereas artificial sea water (Protocols, 2012) modified with NaHCO$_3$ (0.2 g/L) was used for SS304. BCB was prepared by combining equal volume of 100 mM NaHCO$_3$ and Na$_2$CO$_3$ and the desired pH was adjusted using 1M hydrochloric acid (HCl). Pure pyocyanin (Sigma Aldrich) was used in all the experiments and the stock solution was prepared with acetone and di-ionized water as prescribed.

**Table 4.1.** The composition (weight %) of steels used in this study

<table>
<thead>
<tr>
<th>Element</th>
<th>AISI 1030 Composition (wt%)</th>
<th>SS 304 Composition (wt%)</th>
</tr>
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<tr>
<td>C</td>
<td>0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>Mn</td>
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<tr>
<td>Si</td>
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<td>1.00</td>
</tr>
<tr>
<td>P</td>
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<td>S</td>
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<td>0.030</td>
</tr>
<tr>
<td>Cr</td>
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<tr>
<td>Ni</td>
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<td>9.25</td>
</tr>
<tr>
<td>Mo</td>
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<td>-</td>
</tr>
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</table>
4.2.2. Polarization curve

Polarization curve analysis was carried out to evaluate the efficiency of PYO in accepting electrons from indium tin oxide (ITO) and steel compared to oxygen. ITO coated glass slide (working area 13.5 cm$^2$), SS304 (working area 1 cm$^2$) and CS (working area 2 cm$^2$) were used as the working electrode (WE) whereas a graphite rod (4 mm in diameter and 105 mm in length) and a silver-silver chloride (Ag/AgCl) electrode (MF-2052, BASi, USA) served as the counter (CE) and reference electrode (RE), respectively. The experiment was performed in a single chamber membrane less cylindrical reaction vessel having 15 mL electrolyte solution where the counter (CE) and the reference electrode (RE) shared the same electrolyte. The potential of the WE was gradually shifted within a defined potential range (-0.2V to -0.5V) in both pure and modified (with 100 µM PYO) electrolyte solution and the generated cathodic current was recorded. For SS304, the corrosion rate at a specific potential was calculated from the cathodic current generated at that potential using the formula, Corrosion rate (mmy$^{-1}$) = (11.65×Icorr) (CorrosionDoctors, 2018). Where, Icorr = cathodic current density generated at certain potential (mA/cm$^2$). Here, the potentiostatically applied potential was used to determine the Icorr value.

4.2.3. Electrochemical setup and operation

The electrochemical experiments were performed in a modified Twin-WE setup as previously described (Chapter 3). The WE chamber was generated by sandwiching a non-conductive perspex frame (2 mm thick) in between two WEs to load the operating electrolyte. The inlet, outlet and the ion migration channels were generated according to the procedure as previously mentioned and the same type of electrodes were used as the CE (graphite rod) and RE (Ag/AgCl) (Chapter 3, section 3.2.4). The WE chamber was loaded with the operating electrolyte through the inlet channel using a syringe and placed
in a beaker containing ASW (50 mL) solution where the RE and CE were also mounted at fixed locations. Thereafter, all the three electrodes were connected to a custom-made potentiostat and the experiments were operated and monitored by an automated LabVIEW™ software program interfaced with a computer through a data acquisition card (LabJack U12™). All the potentials mentioned in this chapter were quoted against Ag/AgCl reference electrode (+0.210 V vs the standard hydrogen electrode, SHE).

4.2.4. Pyocyanin mediated redox system between stainless steel and ITO
The capability of PYO to establish a redox system for repeated electron extraction from steel was evaluated by using the Twin-WE electrochemical cell described above with two active WEs. One of the WEs was SS304 whereas the other WE was indium tin oxide (ITO) both having the same working area of 13.5 cm\(^2\). The two WEs were facing to each other, creating the WE chamber that contained 1.3 mL of ASW modified with OxPYO (200 µM) as the operating electrolyte. After placing the WE chamber in 50 mL ASW containing beaker, the ITO electrode was poised at 0 V (oxidizing potential for PYO) while the potential of the SS304 working electrode was changed stepwise by 0.1 V per step within the range between -0.2 V and -0.5 V. At each step change, a fixed waiting time (8 min) was maintained to allow any produced RedPYO (by the SS304) to diffuse towards the ITO electrode. The generation of an anodic current at the ITO electrode would thus be a result of a re-oxidation of the RedPYO.

The simultaneous electron accepting and donating capability of PYO between the steel electrode and the ITO electrode was further confirmed by applying the Twin-WE approach as previously described (Hassan et al., 2017). Briefly, the SS304 electrode in the system was used as the reducing electrode (poised at more negative potential than the other electrode) and an ITO electrode served as the oxidizing electrode. A “voltage
window” of 0.1V was maintained between the two electrodes and the “voltage window” was gradually shifted stepwise within 0 V to -0.55 V range by 0.025 V. The waiting time for each step was 4 minutes to allow the generation of a stable current. The generation of a current difference peak (difference between the current of two WEs) is indicative of continuous oxidation and reduction reaction of PYO between the ITO and SS304, respectively.

4.2.5. Effect of oxygen on PYO reduced by steel

The re-oxidation of the RedPYO (reduced by steel) by oxygen was tested electrochemically using the electrochemical setup described above, but instead of two, only one WE was used. The WE chamber was generated by sandwiching a non-conductive perspex frame (2 mm thick) between a SS304 coupon (1 cm² working area) and a non-conductive glass slide. Modified ASW solution containing different concentrations (50, 100 and 200 µM) of PYO and ASW solution without PYO was used as the test and control electrolyte, respectively. The operating electrolyte (1 mL) was loaded inside the WE chamber and the chamber was placed vertically in a beaker containing 50 mL ASW. The SS304 WE was poised at -0.4V to facilitate the reduction of PYO and the cathodic current was recorded. After a stable, low cathodic current was reached (which signified complete reduction of the PYO), air was introduced into the WE chamber to determine the effect of air oxygen on cathodic current. The effect of oxygen was further verified by alternating the air and nitrogen (N₂) gas flow inside the WE chamber for 100 µM pyocyanin containing ASW.

The effect of oxygen on the biologically reduced PYO (reduced by PA) was also investigated by following the above-mentioned procedure. Bio-reduced RedPYO was generated by adding 0.1 mL washed (with ASW) PA cells (pre-grown aerobically in 50%
Luria Bertani broth for 24 hours at 35°C under shaking condition) into a test tube containing 5 mL OxPYO (100 µM) in ASW. The test tube was then purged with N₂ for 5 minutes and was sealed with a butyl rubber bung. After the completion of reduction (as indicated by change in PYO colour from blue to colourless), PA cell free RedPYO was generated by passing the PA containing RedPYO through a membrane filter (pore size 0.22 µm) using a syringe fitted with needle under anaerobic condition. The PA cell free RedPYO was added to the WE chamber and the current was monitored at WE potential of -0.4 V for 4 minutes. Thereafter, air was purged inside the WE chamber and its effect on the cathodic current was recorded.

**4.2.6. Open circuit potential (OCP) monitoring under PYO exposed condition**

Two carbon steel electrodes (working area 1.27 cm²) were used in this experiment since its OCP in BCB (less than -0.4 V) is negative enough to reduce OxPYO. Only one side of the electrode was active and the electrodes were treated following the procedure mentioned in section 2.1 prior to the experiment. Two cylindrical vessels (height 5 cm and diameter 2.7 cm) containing 10 mL BCB (100 mM) were used to perform the experiment where a continuous supply of air was maintained to create a fully oxygen saturated environment. The electrodes were placed separately in the two reaction vessels and the OCP of the electrodes were recorded over time against a silver-silver chloride (Ag/AgCl) reference electrode (MF-2052, BASi, USA). The electrolyte of both the reaction vessels was replaced with fresh electrolyte after a steady OCP was obtained and the OCP of the same steels were monitored in new electrolyte for one hour. Thereafter, the steels were placed in another two different fresh electrolyte containing reaction vessels and one of the reaction vessels was spiked with OxPYO (final concentration 100 µM). The changes in OCP was monitored for one hour and the dissolved iron
concentration was evaluated for all spent electrolytes which was exposed to steel for one hour. Corrosion rate (mmy$^{-1}$) from the dissolved iron concentration was calculated from the following formula:

\[
\text{Corrosion rate (mmy}^{-1}\text{)} = \frac{C \times V}{d \times A \times t \times 87600}
\]

Where, \(C\) = Dissolved iron concentration (g/L); \(V\) = Sample volume (L); \(d\) = Steel density (g/cm$^3$); \(A\) = Exposed area (cm$^2$) and \(t\) = exposure time (hour).

### 4.2.7. Dissolved iron quantification

The concentration of the dissolved iron in solution was evaluated using atomic absorption spectrometer (55 AA, Agilent Technologies). Samples were diluted with 1M HCl (Sample : HCl = 50:50) to dissolve any suspended iron-oxide particles in the solution. Thereafter, the acidified samples were analyzed under the following operating parameters: wavelength 248.3 nm, slit width 0.2 nm, flame air acetylene, lamp current 5 mA.
4.3. Results

4.3.1. PYO can accept electrons more efficiently than oxygen from steel

OxPYO has previously been shown to accept electrons from different prokaryotic and eukaryotic living cells (Chapter 3). PYO can penetrate inside the living cells where it oxidizes cellular NADH and diffuses out of the cell in reduced form (RedPYO). *Pseudomonas aeruginosa* can oxidize this RedPYO in the presence of oxygen and this PA catalyzed oxidation of RedPYO indicates that RedPYO can be an electron donor for the respiration of PA.

The corrosion of steel is triggered by a loss of electrons to suitable oxidizing agents such as oxygen (rust formation). Microbial substances have also been shown to shuttle electrons away from steel, leading to anaerobic microbially influenced corrosion (MIC). However, the transfer of electrons from corroding steel to oxygen has never been shown to be catalyzed by bacterial mediators. The known involvement of PA (Jia et al., 2017a; Li et al., 2017; Li et al., 2016) in microbially influenced corrosion (MIC) validates the investigation of a potential role of OxPYO in extracting electrons from steel. In principle, the exposure of OxPYO to a steel with an open circuit potential (OCP) less than the mid-potential of PYO is expected to cause electron loss of the steel to OxPYO.

The capability of OxPYO to accept electrons from a suitably poised electrode was investigated by monitoring the cathodic current generated by an ITO electrode exposed to OxPYO (in phosphate buffer; PBS). At electrode potentials between -0.3 V and -0.5 V (reducing potential for PYO) OxPYO generated up to 15 times higher cathodic currents density than the oxygenated control (PBS) (Figure 4.1). This shows that, at sufficiently low electrode potential (lower than the mid potential of PYO), the ITO transferred electrons to OxPYO more rapidly than to oxygen.
Chapter 4. Pyocyanin accelerates Pseudomonas aeruginosa associated aerobic corrosion

Figure 4.1. Polarization curve plot for ITO electrode in phosphate buffer (100 mM; pH 7.3). The plot was generated in only PBS (dashed line) and PBS amended by 100 µM PYO (solid line) under oxygen saturated environment.

It is conceivable that OxPYO could also accept electrons from steel at rates higher than oxygen as long as the steel surface was at an electrode potential (open circuit potential) sufficiently negative to reduce OxPYO. To test whether a suitably reduced stainless steel electrode uses OxPYO in preference to oxygen as electron acceptor, polarization curves with SS304 were recorded in the presence and absence of PYO under continuous oxygen supply. As expected, with decreasing potentials, the cathodic current increased regardless of the presence of PYO (Figure 4.2.A). However, at potentials more negative than -0.3 V, OxPYO was a more effective electron acceptor than oxygen, suggesting it could potentially be a corrosive agent under aerobic condition. At a potential of -0.4 V, which is about the open circuit potential of carbon steel, OxPYO enabled a four times higher cathodic current density than that was caused by oxygen alone.
The corrosion rate calculated from the cathodic current density at -0.4 V was 0.021 mm/year (mmy⁻¹) for oxygen reduction. The presence of OxPYO under the same condition increased the corrosion rate to 0.08 mmy⁻¹. This OxPYO influenced corrosion rate is about three times more than that observed with PA biofilm exposed 2707 hyper-duplex stainless steel (0.027 mmy⁻¹) after 7 days (Li et al., 2016). This finding indicates that PYO could be more efficient than oxygen in oxidizing steel at this potential and could be a potential mechanism by which PA containing biofilms cause corrosion.

The increase in cathodic current density in the presence of OxPYO compared to the presence of only oxygen was also observed with CS in bicarbonate buffer (BCB). Cathodic current was only generated in the presence of OxPYO but not in OxPYO free BCB (Figure 4.2.B). Based on the cathodic current density at -0.4 V, the corrosion rate was estimated to be 0.26 mmy⁻¹ which is 3 times more than that recorded for SS304. This result indicates that in exposure to OxPYO, CS would give electrons away faster than SS304 at -0.4 V and thus, CS would be more prone to be corroded by OxPYO than SS304.
Chapter 4. Pyocyanin accelerates Pseudomonas aeruginosa associated aerobic corrosion

Figure 4.2. Effect of 100 µM OxPYO (solid line) on current density at different steel potentials (Polarization plot) against OxPYO free electrolyte (dashed line) for A. SS304 in artificial sea water (pH 7.8) and B. carbon steel in bicarbonate buffer (pH 7.5). The electrolyte was continuously purged with air to maintain an oxygen saturated condition.

4.3.2. PYO can establish a redox system between steel and suitable electron acceptor

The above tests have shown an effect of PYO on cathodic current densities but have not demonstrated the capability of PYO to act as a continued electron mediator from the cathodic surface to a suitable oxidant. The capability of PYO to establish a reversible electron accepting and donating system was tested using the Twin-WE electrochemical cell (as described in Chapter 3, section 3.2.4). A PYO redox system was generated by using a steel (SS304, poised at -0.4 V) electrode as the electron donor for OxPYO and an ITO electrode (poised at 0 V) for oxidizing the RedPYO. The OxPYO in the ASW-electrolyte was reduced by SS304 electrode at different potentials and the generation of the respective oxidizing current on the ITO electrode was monitored. Result show the generation of oxidizing current on the ITO electrode only when the SS304 electrode was poised at sufficiently low potentials (lower than -0.3 V) to enable the reduction of OxPYO (Figure 4.3.A). Results show that PYO mediated
a continued electron transfer from a cathodic steel surface via diffusion to an oxidizing agent.

The reversible oxidation and reduction property of PYO was further confirmed by applying the “voltage window” shifting method within a microscale (0.3 µL operating sample) thin layer (250 µm) system in Twin-WE cell where OxPYO (200 µM) was used as the operating mediator. Based on the principle of “voltage window” shifting method (Chapter 2), the existence of a redox active compound between the two WEs will be indicated by the generation of a current difference peak at the mid-point of the compound. The generation of the current difference peak represents maximum oxidation-reduction reaction in between two WEs and in principle, this happens only when the potential of one of the WEs poised below and other above the mid-potential of the mediator. As expected, a distinct current difference peak (the difference in currents generated in two WEs, indicative of the electron flow between them) at -0.4 V was developed which is indicative of the presence of pyocyanin between two WEs (Figure 4.3.B). The difference in current between two WEs is the result of the reduction of OxPYO on SS304 electrode and the subsequent oxidation of the RedPYO on the ITO electrode. Generation of such redox system in the presence of PYO is an indication of the simultaneous electron accepting and donating capability of OxPYO from the steel and ITO electrode, respectively.
Chapter 4. Pyocyanin accelerates Pseudomonas aeruginosa associated aerobic corrosion

4.3.3. PYO can accelerate electron transfer from steel to oxygen

It is evident from the above results that OxPYO can efficiently accept electrons from the steel surfaces at potentials less than -0.3 V while being oxidized by an oxidant such as an anode. To test if the RedPYO generated from a steel cathode at -0.4 V can also be re-oxidized by oxygen (instead of ITO electrode at 0 V), air was purged inside the WE chamber containing RedPYO (pre-reduced by SS304 at -0.4 V) and the cathodic current was monitored. Result showed an immediate increase in cathodic current after purging the air which is indicative of the re-oxidation of RedPYO by oxygen. The newly generated cathodic current was stable as long as the air flow was maintained inside the WE chamber (Figure 4.4.A). This test was repeated for different concentrations of PYO and the intensity of the cathodic current generated after introducing the air was found to approximately proportional to the concentration of the PYO present (Figure 4.4.B).

Figure 4.3. A. Current generated in ITO electrode (at 0 V) due to the oxidation of RedPYO generated by poising the SS304 at potentials -0.2 V (black line), -0.3 V (brown line), -0.4 V (blue line) and -0.5 V (red line) in the presence of pure PYO (200 µM) B. Twin-WE plot (“voltage window” 0.1 V and step size 0.025 V) in the presence of pure PYO (200 µM) where SS304 was served as the reducing electrode and ITO was the oxidizing electrode.
Figure 4.4. A. Effect of oxygen on cathodic current generation in the presence 0 µM (Red line), 50 µM (brown line), 100 µM (green line) and 200 µM (blue line) pyocyanin (PYO) concentrations in artificial sea water (ASW). After inserting the sample into WE chamber, the WE (SS304) was poised at -0.4 V (vs. Ag/AgCl) to reduce the PYO. After the development of a stable cathodic current, air was purged inside the WE chamber and the resulted current was recorded. B. Relationship between cathodic current and corresponding PYO concentration.

The role of oxygen in re-oxidizing the RedPYO was confirmed by alternating the flow of air and nitrogen gas (N_2) inside the WE chamber. Result showed an increase in cathodic current only when air was supplied inside the chamber whereas under nitrogen purging condition, the cathodic current decreased over time (Figure 4.5).
Figure 4.5. The effect of oxygen and nitrogen on the cathodic current generated by SS304 at -0.4 V for artificial sea water (ASW) containing 100 µM pyocyanin.

The re-oxidation of RedPYO by oxygen appears to be in contrast to our previous finding (Chapter 3) where RedPYO was found to be non-oxidizable by O₂ unless catalyzed by living cells. Thus, the previously observed resistance of biologically reduced PYO towards oxidation by O₂ was re-evaluated by replacing electrochemically reduced PYO with biologically reduced PYO (obtained by incubating pure OxPYO with washed PA cells in the absence of oxygen). No significant change in cathodic current (less than 3 µA) was observed after purging air into the chamber (Figure 4.6). This is in striking contrast to the effect of oxygen addition to electrochemically reduced PYO where a current jump of 30 µA was recorded (Figure 4.5). The reason for a different behavior of electrochemically and biologically reduced PYO with respect to oxygen is unclear and discussed below.
4.3.4. PYO increases the dissolution of iron under oxygenated condition

In combination, the above findings suggest OxPYO is a more efficient electron acceptor than oxygen in extracting electrons from the steel surfaces. Further, the capability of electrochemically reduced PYO to be rapidly re-oxidized by oxygen in the presence and potentially also in the absence of PA cells, defines it as a potential catalyst for electron transfer from cathodic steel surfaces to oxygen. In principle, then OxPYO is expected to be a corrosive agent for the aerobic rusting process of steel.

The effect of OxPYO on the undisturbed aerobic corrosion of iron was evaluated by exposing a CS to OxPYO in the presence of oxygen under open circuit condition. The presence of OxPYO increased the corrosion rate of CS about two-fold compared to oxygen alone (Figure 4.7). The immediate increase (more positive) of open circuit potential (OCP) after the addition of OxPYO supports the corrosive effect of PYO as it is indicative of the loss of electrons from the steel.
Chapter 4. Pyocyanin accelerates Pseudomonas aeruginosa associated aerobic corrosion

Figure 4.7. The effect of OxPYO (100 μM) on open circuit potential (OCP) and dissolution of iron from steel. The experiment was conducted by exposing carbon steels (CS) to OxPYO free and OxPYO containing bicarbonate buffer under oxygen saturated condition for 1 hour. Samples were then analyzed to quantify the amount of dissolved iron using atomic absorption spectrometer (AAS).

Visual inspection of PYO exposed steel specimen showed strong discolouration by corrosion products (orange pits, blue tinge of surface) while only minor orange spots were observed on the control sample which could point to underestimated corrosion product determination and pitting corrosion.
4.4. Discussion

This study demonstrates the potential role of a biological electron mediator in accelerating aerobic corrosion by factor between 2 and 19.6-fold (Table 4.2). The addition of OxPYO under oxygenated condition caused significant increase in the corrosion rate of steels measured both from the cathodic currents and amount of dissolved iron. At a potential of -0.45 V, the calculated OxPYO affected corrosion rate was about 3 times lower for SS304 than the measured (by iron dissolution) corrosion rate for CS at open circuit potential at -0.46 V to – 0.41 V.
Table 4.2. The list of corrosion rates (mmy⁻¹) calculated from the cathodic currents and the amount of dissolved iron released from the CS. ITO and SS304 electrodes were used to monitor cathodic currents at different potentials in the presence and absence of OxPYO (100 µM). CS (in OCP setup) was used to determine the amount of dissolved iron released to the oxygenated electrolyte in the presence and absence of OxPYO (100 µM).

<table>
<thead>
<tr>
<th>Cathode type</th>
<th>Potential (V)</th>
<th>Current density with oxygen (mA/cm²)</th>
<th>Current density with oxygen and OxPYO (100 µM) (mA/cm²)</th>
<th>Calculated corrosion rate with oxygen (mmy⁻¹)</th>
<th>Calculated Corrosion rate with oxygen and OxPYO (mmy⁻¹)</th>
<th>Factor of aerobic corrosion enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITO</td>
<td>-0.3</td>
<td>0.0000066</td>
<td>0.00052</td>
<td>7.6 × 10⁻⁵</td>
<td>0.0006</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>-0.4</td>
<td>0.00097</td>
<td>0.0013</td>
<td>0.0011</td>
<td>0.0131</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>-0.5</td>
<td>0.0017</td>
<td>0.00334</td>
<td>0.00198</td>
<td>0.0389</td>
<td>19.6</td>
</tr>
<tr>
<td>SS304</td>
<td>-0.3</td>
<td>0.00446</td>
<td>0.00108</td>
<td>0.0051</td>
<td>0.0125</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>-0.4</td>
<td>0.0018</td>
<td>0.0075</td>
<td>0.0209</td>
<td>0.0873</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>-0.45</td>
<td>0.00302</td>
<td>0.01617</td>
<td>0.0351</td>
<td>0.1884</td>
<td>5.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Steel type</th>
<th>Condition</th>
<th>Change in OCP (V)</th>
<th>Dissolved iron (mg/L)</th>
<th>Measured Corrosion rate (mmy⁻¹)</th>
<th>Factor of aerobic corrosion enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>Oxygen</td>
<td>-0.460 to -0.459</td>
<td>4.02</td>
<td>0.353</td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>Oxygen + OxPYO (100 µM)</td>
<td>-0.462 to -0.411</td>
<td>8.24</td>
<td>0.723</td>
<td>2.04</td>
</tr>
</tbody>
</table>

Corrosion rates obtained from cathodic current

Corrosion rates calculated from dissolved iron
The effect of externally added electron mediators (FAD and riboflavin) in enhancing the corrosion rate of PA (Jia et al., 2017b) and sulfate reducing bacteria (SRB) (Zhang et al., 2015) exposed steels were studied previously under anaerobic condition. FAD and riboflavin didn’t show significant corrosive property as individual species. The addition of these electron mediators caused a maximum of 27% increase in corrosion rate (from 0.136 mmy$^{-1}$ to 0.173 mmy$^{-1}$ and 0.155 mmy$^{-1}$) in PA exposed steel while a two fold increase in corrosion rate was observed for SRB exposed stainless steel (0.012 mmy$^{-1}$ to 0.022 mmy$^{-1}$ and 0.028 mmy$^{-1}$). Interestingly, our results suggest that OxPYO itself has steel oxidizing property and the re-oxidation of RedPYO by oxygen can sustain aerobic corrosion.

This finding encourages future investigations on other microbial mediators characterized by mid-potentials similar to PYO such as AQDS (MP = -0.39 V) (Benz et al., 1998) which could also have similar steel oxidizing property. AQDS and humic substances have been reported to accelerate the MIC associated electron transfer processes in the presence of bacteria (Nevin & Lovley, 2000). However, their individual ability to extract electrons from the steel surface has not been investigate yet.

The oxidation of electrochemically generated RedPYO by oxygen (Figure 4) seems to be in contrast to the findings from the previous chapter where RedPYO was found non-oxidizable by oxygen. This contrasting phenomenon could be explained by three redox states of PYO namely oxidized, monovalently reduced and divalent reduced (Goyal & Manoharachary, 2014; Hassan & Fridovich, 1980). It could be possible that any of these two reduced forms of PYO (most probably the monovalently reduced species) can reversibly react with oxygen while the other one is not oxidizable by oxygen.
Chapter 4. *Pyocyanin accelerates Pseudomonas aeruginosa associated aerobic corrosion*

The mechanism of OxPYO in accelerating steel corrosion can be attributed by its cathodic stimulation property. OxPYO driven cathodic stimulation (electron loss) can be explained by the immediate shift in OCP to more positive value after the addition of OxPYO (Figure 4.7). This phenomenon has been reported previously where PA culture maintained a stable and more positive OCP (-0.193 V) of a stainless steel for 14 days compared to the abiotic control (-0.407 V) (Li et al., 2016). The stability of the OCP in OxPYO (in this study) or PA culture (Li et al., 2016) exposed steel can be attributed due to the existence of simultaneous reduction and oxidation of OxPYO by steel and oxygen, respectively.

The capability of OxPYO to extract electrons from steel could explain the role of this electron mediator in PA biofilm exposed steel corrosion. Since RedPYO is oxidizable by PA in the presence of oxygen (Chapter 3), steel oxidizing capability of OxPYO could offer PA as an alternative electron source for respiration.

Electrochemical reduction of oxygen by an electrode colonized by PA cells has already been studied and it is reported that the electron transfer rate was enhanced four times due to the presence of PA cells (Cournet et al., 2010). However, their results were estimated from cyclic voltammograms and the direct corrosion rates were not mentioned. The presence of PA cells on the electrode didn’t cause any significant increase in the cathodic peak current, but interestingly, the cathodic current was generated at more positive potential (at -0.2 V) than that generated with suspended PA cells (at -0.4 V). Although they concluded that the presence of PYO is not necessary for this cathodic electron transfer, the possibility of the involvement of PYO was also not excluded.

Mediators have been shown to enhance microbial anaerobic corrosion (Jia et al., 2017b; Zhang et al., 2015). However, compared to the MIC in the presence of anaerobic electron
acceptors (nitrate, sulfate), the stimulating effect was between 15% to 30%. This study reports the individual effect of an electron mediator in stimulating aerobic corrosion between 200% to 1900%. Compared to the literature available in this study, it can be concluded that PYO is a unique electron mediator which can cause faster corrosion than oxygen by mediating the transfer of cathodically derived electrons to oxygen. This study also suggests that an aerobic bacterium can benefit from corrosion by using steel as an electron donor for their energy conservation.

Proposed mechanism
The findings of this study lead to portray a mechanism for PYO mediated aerobic steel corrosion. Development of PA biofilm in exposure to a steel (having OCP more negative than -0.244 V) will create a suitable condition for OxPYO to extract electrons from the steel. The RedPYO generated in exposure to the steel will then be available to get oxidized. Re-oxidation of the resultant RedPYO can be occur both by oxygen (where available) and PA cells (in the biofilm exposed to air). This will result in the establishment of a PYO mediated electron transfer mechanism from the steel to oxygen. PA cells could profit from this condition by driving the electron flow from the RedPYO to oxygen for ATP generation (Figure 4.8).

The faster electron transfer capability of PYO or PA (by oxidizing RedPYO) from the steel to oxygen would accelerate the cathodic half reaction. Acceleration of the cathodic reaction will consequently accelerate anodic reaction and thereby the corrosion process.
Figure 4.8. Schematic of the proposed “PYO mediated aerobic corrosion acceleration mechanism”.

4.5. Conclusion

This study demonstrates the role of pyocyanin as an effective cathodic stimulant in enhancing aerobic steel corrosion. Our result encourages further investigation on the potential role of different microbial redox active metabolites individually or in concert with their producers in influencing metal corrosion processes.
Chapter 5: Conclusion and outlook

This study makes a scientific contribution to the measurement and functionality of bacterial redox mediators. The outcomes of this study are the development of a new technique for characterizing electron mediators and the discovery of mediator dependent energy extraction from living cells as well as corroding iron.

5.1. New method for characterizing electron mediators with Twin-WE cell

An ideal electrochemical cell consisting of two working electrodes was developed for evaluating microbial redox system and the application of this electrochemical cell has been shown for the precise characterization of electron mediators. The suitability and sensitivity of the proposed “redox gradient” shifting method encourages its application in characterizing electron mediators in complex microbial systems such as anaerobic digester, rumen, human gut etc. The sensitivity and versatility of the Twin-WE system can be improved by proper modification of the electrodes (e.g. coating with electrochemically active chemical or biological compounds) and the integration of more sensitive current monitoring device. The unique property of being equipped with two WE\textregistered{s}, the Twin-WE cell can be used to conduct some potentially interesting investigations on microbial metabolism as mentioned below.
5.1.1. Can bacteria grow only on redox gradient?
In nature, the driving force for respiration via electron transport phosphorylation is the redox gradient between electron donor and acceptor. Bacteria utilize this redox gradient to catalyze the electron transfer reaction through their electron transport chain for generating ATP. Since it is known that electroactive bacteria can utilize electrodes both as electron donor and acceptor depending on their potential, it would be interesting to test if bacterial growth is possible under a condition where electrodes will serve as both electron donor and acceptor instead of organics and chemical electron acceptors.

5.1.2. How does the interspecies electron transfer occur in complex microbial systems?
Interspecies electron transfer (IET) processes have been reported to play the key role in complex microbial systems such as anaerobic digesters where multistep electron transfer reactions occur amongst the species to transfer the electrons from the electron donor to the terminal electron acceptor. The physical contact of the conductive cellular components (e.g. c-type cytochromes and nanowires) between cells allows the flow of electrons within the biofilm where IET prevails. The developed twin-WE could be an effective tool to evaluate the mechanism of electron flow within different species of the biofilm. The analysis can be performed by placing a IET conducting biofilm in between the twin WEs and create defined redox condition on either side by controlling the potentials of the two WEs. Variations in the redox condition could be made to bring changes in the mode of electron transfer within the biofilm. Monitoring of the electron flow upon changes in the redox condition could bring some new understanding on the following questions.

1. What is the minimum potential gradient required to drive the IET within a biofilm?
2. Can the natural electron donors (organics) and acceptors (e.g. CO₂, O₂ or NO₃⁻) be replaced with suitably poised electrodes?

3. Does the replacement of either natural electron donor or acceptor by electrodes enhance the overall IET rate?

This controlled redox environment within a biofilm is expected to enable a better understanding of biofilms in situations such as in gut environment, marine sediment, microbially influenced corrosion etc.

5.2. Electron mediator driven energy extraction

A novel energy (electron) harnessing function of an electron mediator has been proposed in this study. The tested electron mediator (PYO) has been shown to take electrons away from both biological (e.g. living cells) and chemical sources (e.g. steel). This electron harnessing mechanism depends on the reversible reduction and oxidation of PYO by reducing sources and *P. aeruginosa*, respectively. The benefit of PYO to its producer (*P. aeruginosa*) is its ability to act as an electron donor for PA to respire on both oxygen and nitrate. The PYO mediated electron extraction from living cell resembles the interspecies electron transfer. However, here, one species acquires the reducing equivalents (NADH) of another cell, thus playing an energy parasitic role. Based on these findings of this study, the following investigations could be performed to elucidate underlying mechanisms.
5.2.1. What controls the oxidation of RedPYO by oxygen?
PYO reduced by exposure to living cells was stable in the presence of oxygen, while electrochemically reduced RedPYO readily reacted with oxygen to become oxidized. This contrasting finding is very interesting, and the possible reason could be the difference in the reduction processes (e.g. biological versus electrochemical reduction).

The oxidizing property of RedPYO could be explained by the three redox states of PYO namely oxidized, mono-valently reduced and divaletly reduced. Although it is believed that reduced forms of PYO are readily oxidizable by oxygen, it was not mentioned if both reduced forms of PYO exhibit the same phenomenon. Thus, the evidence of the existence of a non-oxygen-sensitive form of RedPYO (produced in exposure to living cell) necessitates investigation on the nature of the oxygen sensitivity of the two reduced forms of PYO.

5.2.2. Is PYO mediated energy extraction universal for all PA strains?
This study was conducted using a state (Western Australia) pure culture of \textit{P. aeruginosa} (WACC 91). Although it is perceivable that PYO depending mechanisms should be universal for all \textit{P. aeruginosa} strains, it can be confirmed with other PA strains such as PAO1.

5.2.3. Can other electron mediators spontaneously extract electrons from living cells?
PYO is one of the many biologically important electron mediators such riboflavin, AQDS, cysteine, quinone, humic acid etc. These electron mediators have been reported to transfer electrons directly between bacterial cells or bacterial cells and insoluble compounds. Thus, it is worthwhile to investigate if these electron mediators have similar capabilities.
5.2.4. Does pyocyanogenic PA have greater fitness advantage over apyocyanogenic PA?

In principle, the intriguing capability of PYO to act as an energy extractor from living cells for PA should favour pyocyanogenic PA to sustain under carbon source limiting condition. It has been shown in this study that both *E. coli* and yeast cells lose electrons to PYO form their NADH and the resulted RedPYO serves PA as electron donor. This could give PA an energetic advantage over surrounding cells.
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