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Extracellular Electron Transfer in Phototrophic Microbial Communities

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Abstract

Microbial solar cells (MSCs) are bioelectrochemical systems where photosynthetic microorganisms catalyse solar energy conversion to electricity through oxidation of water/organic carbon at solid-state electrodes, coupled to reductive reactions at a counter electrode, typically oxygen reduction to water. MSCs have potential for niche applications, particularly generation of off-grid electricity to power devices in remote areas, including biosensors. The key advantage over other solar technologies is that steady electrical output can be produced 24 hours a day.

This thesis explores MSCs as a potential technology, by studying the underlying principles of phototrophic electroactivity and by engineering high-throughput electrodes.

The usage of microbial consortia rather than pure cultures was chosen on the basis of higher electrical output and higher robustness to fluctuating process conditions. This thesis studies the process of extracellular electron transfer (EET) using three-electrode photoelectrochemical cells, inoculated with environmental cultures from fresh and saltwater sources, at poised working electrode potential of +0.6 V vs. SHE under day/night cycles.

The following research objectives were sought: (i) to investigate whether EET is a common feature in naturally occurring phototrophs; (ii) to elucidate the link between photosynthetic oxygen and the observed phenomenon of reversible currents; (iii) to elucidate the metabolic reasons for the occurrence of EET in mixed phototrophic communities; (iv) to enhance performance towards creation of MSC devices.

The natural occurrence of EET in environmental phototrophic microorganisms was investigated for several fresh and seawater cultures. No electron flux to electrodes was observed with any fresh water cultures, and added mobile redox mediator (riboflavin) was required to establish EET. Microbial communities in two identified enriched fresh water cultures showed a majority of microalgae. It appears instead that EET is ubiquitous in seawater environments, with cyanobacteria dominance in two tested biofilms. Within the electrochemically active seawater cultures a link was found between the existence of a dual microbial community (cyanobacteria and gamma-proteobacteria), and the generation of fully reversible current, anodic at night and cathodic during daytime. A consortium without gamma-proteobacteria exhibited negligible daytime output.

The detrimental effect of photosynthetically evolved oxygen on EET was investigated in the presence of riboflavin upon illumination of a freshwater-mixed phototrophic culture. The working electrode compartment produced an electrical current in response to day/night cycles over 12 months of operation, generating a maximum current density of 17.5 mA m^{-2} during the night phase, and a much lower current of approximately 2 mA m^{-2} during illumination. The cause of lower current generation under light exposure was found to be the high rates of re-oxidation of reduced riboflavin by the oxygen produced during photosynthesis. A similar detrimental effect was observed in seawater cultures without mediator addition.

The metabolic reason for EET in phototrophs was studied using previously enriched and electrochemically active marine microbial biofilms dominated by cyanobacteria. Inorganic carbon (Ci) was supplied by addition of $NaHCO_3$ to the medium and/or by sparging CO_2 gas. At high Ci conditions, anodic EET was observed only during the night phase, indicating the occurrence of a form of night-time respiration that can use insoluble electrodes as terminal electron acceptors. At low or no Ci conditions however, EET also occurred during illumination, suggesting that, in the absence of their natural electron acceptor, some cyanobacteria are able to utilise solid electrodes as an electron sink. This may be a natural survival mechanism for cyanobacteria to maintain redox balance in environments with limited CO_2 and/or high light intensity.

The performance enhancement was conducted by co-immobilisation of mat-building seawater photosynthetic consortia and polymeric redox mediators (polymeric osmium complexes and polymeric azine mediators, including polymethylene blue and green, polythionine) onto anodes. Importantly, by this co-immobilisation, the previously observed detrimental effect of oxygen to photo current generation disappeared – a higher anodic current was exhibited during the day than at night – with uninterrupted anodic current generation during the day/night cycles. The largest improvement of anodic current outputs was achieved with electrodes immobilised by polymeric osmium complex [Os(2,2'-bipyridine)$_2$(polyvinyl-imidazole)$_{10}$Cl]Cl, to 320 ± 28 mA m^{-2} (64 ± 6-fold) under illumination and 317 ± 29 mA m^{-2} (43 ± 8-fold) at dark, compared with the bare graphite bioelectrode.

This thesis offers an analysis and insight on the biological complexity that drives the electron transfer processes at MSC anodes. It also describes a number of technological

advances that enabled the enhancement of power outputs by one order of magnitude compared to previous work.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Journal publications:

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Darus L, Sadakane T, Ledezma P, Tsujimura S, Osadebe I, Leech D, Gorton L, Freguia S (2015) Redox polymers enable day/night photo-driven electricity generation by mixed seawater consortia. Bioelectrochemistry, in preparation.

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Contributor	Statement of contribution
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(i) Mr Bernardino Virdis from UQ contributed to the conception in Chapter 2 and 3

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Keywords

extracellular electron transfer, photosynthetic evolved oxygen, biophotovoltaics, microbial solar cell, reversible current, cathodic current, excess energy dissipation, mediator, modified electrode surface, photosynthetic mixed culture

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Abbreviations	
ADP^+	Nicotinamide adenine dinucleotide phosphate
BESs	Bioelectrochemical systems
CA	Chronoamperometry
CCM	Concentrating mechanism
CEM	Cation exchange membrane
Ci	Inorganic carbon
CLSM	Confocal laser-scanning microscope
CV	Cyclic voltammetry
Cyt	Cytochrome
DAD	Diaminodurene
DAPI	4′6-diamidino-2-phenylindole
DEET	Direct extracellular electron transfer
DET	Direct electron transfer
DMBQ	2,6-dimethyl-1,4-benzoquinone
DO	Dissolved oxygen
DOC	Dissolved organic carbon
E	Redox potential
EET	Extracellular electron transfer
Fd	Ferredoxin
FISH	Fluorescence in situ hybridisation
GC	Gold Coast
HNQ	2-hydroxy-1,4-naphtoquinone
HRT	Hydraulic retention time
IEET	Indirect extracellular electron transfer
MB	Methylene blue
MFC	Microbial fuel cell
MG	Methylene green
MSC	Microbial solar cell
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NPOC	Non purgeable organic carbon
PC	Plastocyanin
PETC	Photosynthetic electron transport chain

PMFCs	Photosynthetic microbial fuel cells
PQ	Plastoquinone
PS	Photosystems
Q_A	Quinone pool
RETC	Respiratory electron transport chain
ROS	Reactive oxygen species
SC	Sunshine Coast
SHE	Standard hydrogen electrode
TIC	Total inorganic carbon

Chapter 1
Introduction

1.1 Background

Renewable energy sources are those that are consumed at a rate that is similar to the rate at which they are naturally replenished. They are typically derived from solar, geothermal or hydroelectric power. Due to the global issues of fossil fuel depletion and climate change, the development of renewable energy sources has gained momentum (Rosenbaum et al. 2010). The largest contribution to renewable energy originates from solar light (Dieter et al. 2006).

The amount of solar energy that strikes the Earth hourly accounts for approximately 4.3×10^{20}J – approximately equal to the annual energy consumption by human society (Donohue and Cogdell 2006). Considering the abundance of solar energy, studies on solar energy conversion into ready-to-use energy have been intensively performed. At present the most well-known technologies for converting solar energy into electric power are photovoltaics, known for the simple design and installation but still regarded as a relatively expensive technology (El Chaar et al. 2011). Two existing technologies for indirect conversion of solar energy to usable energy are bioethanol production by fermentation of sugary (first generation) or lignocellulosic (second generation) biomass, and biodiesel production by extraction of either plant or algal oil (Angermayr et al. 2009). Both processes rely on solar energy conversion into biomass with relatively low efficiency (<10%, even <5% in practice), compared with a maximum of 30% that can be stored as chemical energy in form of Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) (Melis 2009). Attempts have been directed to sunlight-driven hydrogen production catalysed by photosynthetic microorganism (Angermayr et al. 2009; Ghirardi et al. 2009), but the yield in this process is limited by the photosynthetic reaction kinetics, which are slowed down by the high partial pressure of hydrogen in the media (Rosenbaum et al. 2005). To maintain a low partial hydrogen pressure, Cho et al. (2008) and Rosenbaum et al. (2005) successfully combined the photosynthetic biohydrogen generation with in situ hydrogen oxidation at solid-state electrode in bio-electrochemical systems (BESs).

Some microorganisms are capable of oxidising electron donors, transfer the generated electrons extracellularly through the outer surface of the cell, towards other cells and/or to reduce extracellular electron acceptors *e.g.* mineral oxides. Such process is called

extracellular electron transfer (EET). Devices that utilise the EET capability to capture electrons (generated over heterotrophic respiration on organic matter) at bioelectrodes in bioelectrochemical systems (BESs) has been previously well described, including microbial fuel cells (MFCs) (Logan et al. 2006). When the electrons sources are mainly from light-driven water oxidation by photosynthetic microorganisms, the corresponding device is called microbial solar cell (MSC) (De Schamphelaire and Verstraete 2009), or biophotovoltaic, or photosynthetic microbial fuel cell. MSCs make use of biocatalysts with natural capability of self-repairing and reproduction, hence offer a potentially cheaper catalyst compared to photovoltaics. Moreover, the direct capture of water-derived electrons at electrodes is expected to be more efficient than indirect methods such as biofuel production, which require growth, harvest and processing of biomass such as corn and sugar cane. In the case of MSCs, the photosynthetic organisms are mere catalysts of energy harvest (McConnell et al. 2010; Freguia et al. 2012; McCormick et al. 2015; Strik et al. 2011).

Experiments with MFCs demonstrated that the use of microbial consortia for current generation is advantageous over axenic cultures, as consortia produce higher electrical outputs and have higher resilience to variable physicochemical and process conditions (McCormick et al. 2015). The higher resilience may provide better long-term stability for applications.

Using the most abundant sources of energy (solar light), electron (water), carbon (atmospheric CO_2) (Badura et al. 2011; Peschek et al. 2004) and the superiority of environmental photosynthetic consortia, MSCs have the potential to be a remarkable bioenergy source for a number of niche applications such as the generation of off-grid electricity to power devices in remote areas and biosensors.

1.2 Thesis Scope and outline

In order to open the path toward implementation of MSC devices for electrochemical power production from sunlight catalysed by phototrophic consortia, it is of great significance to understand the natural occurrence of phototrophic microorganisms with EET capability and to capture their biocatalytic capability to create a device for the conversion of sunlight to electricity, which is the overarching goal of this PhD thesis.

This thesis comprises seven chapters. **Chapter 2** consists of a literature review – exploring the information within the photosynthetic electron flow, MSC, EET pathways and metabolisms in cyanobacteria, and electron shuttling redox mediators, leading to identification of knowledge gaps, formulation of research objectives and hypotheses, and providing a brief summary of the outcomes of each objective. Chapters 3-6 consist of the published (3-5) and submitted (6) papers addressing those research objectives. **Chapter 3** describes the first thorough study on how photosynthetically evolved oxygen suppressed light-driven anodic current generation by a fresh water mixed phototrophic culture dominated by algae in the presence of riboflavin, and shows that with a mediator-less system, the phototrophic consortia gave no electrical response to the illumination/dark cycles. **Chapter 4** shows the first link between the phenomenon of fully reversible current – cathodic during the illumination and anodic at dark time – and the dual composition of a marine photosynthetic microbial community, comprising phototrophic cyanobacteria and oxygen-reducing bacteria (gamma-proteobacteria) in mediator-less systems. This chapter also shows how two seawater phototrophic biofilms dominated by distinct cyanobacteria and other bacteria exhibited different electrogenic activity, and shows the role of oxygen in cathodic current generation. **Chapter 5** reports the EET occurrence by marine cyanobacterial consortia which transferred electrons to electrodes in response to reductive stress and delivers some mechanistic aspects including the electron donors and pathways of EET. **Chapter 6** discusses the enhancement of current generation through co-immobilisation of polymeric mediators and mat-building photosynthetic consortia onto carbon electrode surfaces. Finally, **Chapter 7** summarises the insights gained over the PhD candidature and further discusses potential applications based on the achievements during the candidature and the literature. Research ideas are also proposed for further work to take the technology to a mature stage for future applications.

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Chapter 2
Literature review and research questions

This literature review will explore the information within: i) the photosynthetic electron flow, ii) introduction to MSC, iii) occurrence of photosynthetic microorganisms, iv) EET pathways and metabolisms in cyanobacteria, and v) electron shuttling redox mediator. The review is then followed by research questions.

2.1 Photosynthetic electron flow

Photosynthesis is a sustainable, effective and complex process that converts solar energy into chemical energy, occurring in photosynthetic organisms (Yehezkeli et al. 2012). The majority of photosynthetic organisms are oxygenic, which means that they use water as electron donor and a minority are anoxygenic – using sulphide or organic carbon as electron donor (Madigan 2003). In this thesis, the term of photosynthetic/phototrophic organisms refers to oxygenic photosynthetic organisms.

Solar energy conversion in eukaryotic photosynthetic organisms such as green-plants, microalgae and in prokaryotic photosynthetic microorganisms such as cyanobacteria operates over two reactions centres: photosystems 1 (PS1) and photosystems 2 (PS2), which are protein complexes – embedded in the thylakoid membranes. PS1 and PS2 are linked in series and interact through a chain of electron carriers, consisting of enzymes and co-factors (protein complexes) including plastoquinone (PQ), plastocyanin (PC), the cytochrome (Cyt) b6f complex, and ferredoxin (Fd). The electron carriers have the function to transport electrons produced during photosynthetic water oxidation to the terminal electron acceptor nicotinamide adenine dinucleotide phosphate ($NADP^+$). In principle, electrons are transferred from a lower to a higher potential electron carrier.

In PS2, photo-excitation of redox potential (E) – encountered by chlorophyll-based molecule (P680) from its normal state at +1.25 V SHE (Standard Hydrogen Electrode, SHE) to its excited state at −0.58 V SHE – leads to water oxidation to oxygen, proton and an electron flow to the quinone pool (Q_A) (acceptor that stimulates the reduction of PQ). PQ can accept electrons from both PS2 and PS1 and normally transfer the electrons to Cyt b6f, to a soluble electron carrier (PC) on the thylakoid lumen, and further to PS1 (Paumann et al. 2005). In PS1, photo-excitation of redox potential (E) – encountered by

P700 from its normal state +0.43 V to its excited state −1.3 V (SHE) – leads to an electron flow to Fd, ultimately furthering the production of NADPH that activates the Calvin cycle for CO_2 fixation to organic compounds and biomass (Yehezkeli et al. 2012; Badura et al. 2011). Another path – where the electrons are donated from Fd to hydrogen evolving enzyme-hydrogenase – synthesizes molecular H_2. Here protons are the terminal acceptors of the photosynthetically generated electrons (Melis 2009). The intracellular and extracellular electron transfer mechanisms in photosynthetic electron transport chain (PETC) are illustrated in Figure 2.1.

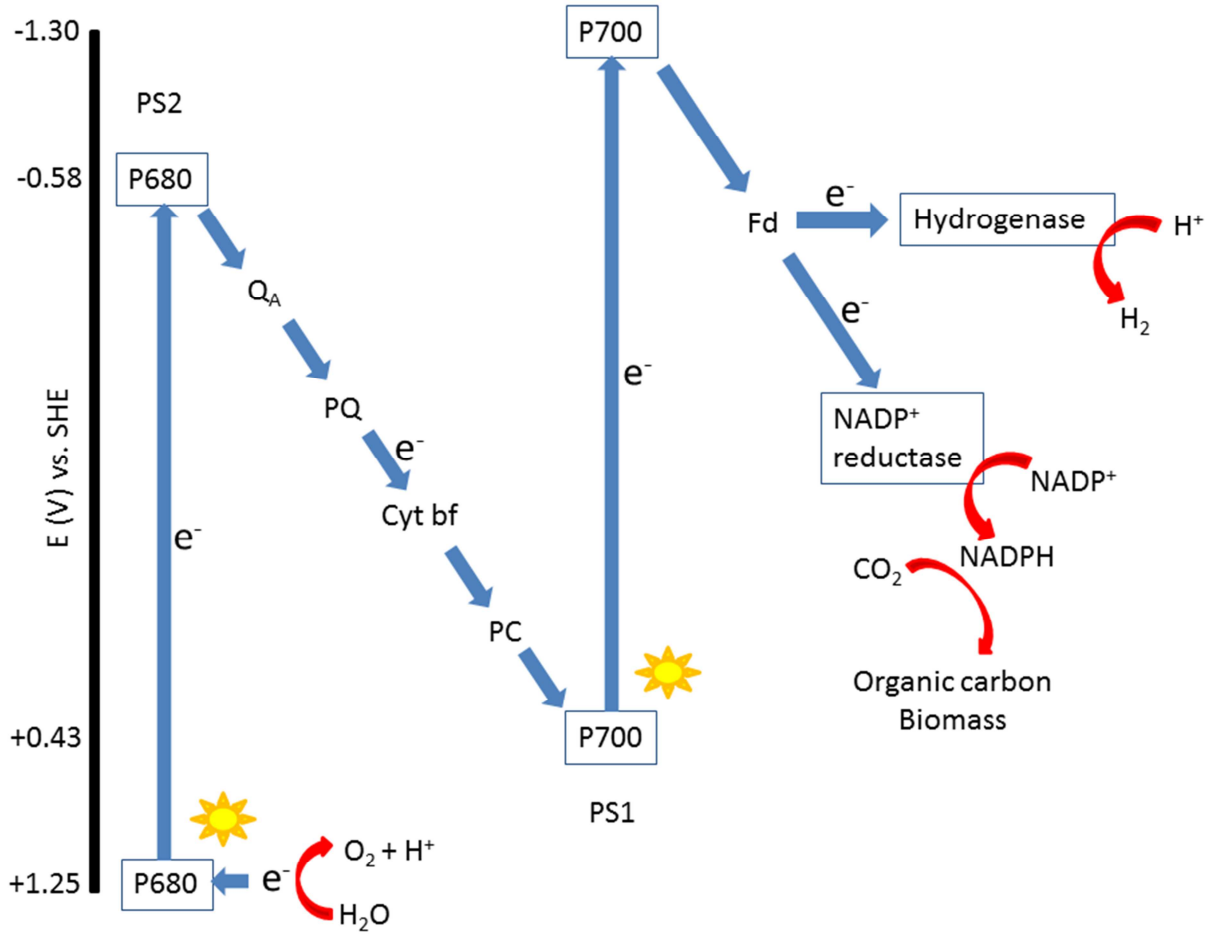

Figure 2.1 Intracellular electron transfer paths in photosynthetic organisms

2.2 Introduction to MSC, configuration and potential application

Photosynthetic water splitting to oxygen, protons and electrons (Equation 1) is the net anodic reaction in MSCs. As described above, under illumination photosynthetic microorganisms produce organic compounds through CO_2 fixation – stored within the cytoplasm (Herrero and Flores 2008) and sometimes excreted outside the cells (Badura et al. 2011). Endogenous and exogenous respiration on organic matter does not appear in

the overall anode reaction. Over the electrical circuit, the electrons are transferred to the cathode, where they reduce oxygen (in the presence of protons) to reform water (Equation 2). In case photosynthetic mixed cultures are used as biocatalyst, net anodic reaction may be a multi-step reaction involving multiple populations when phototrophic and chemo-heterotrophic microorganisms are symbiotically involved.

Net anodic reaction : $2H_2O \rightarrow O_2 + 4H^+ + 4e^-$ (Equation 1)

Net cathodic reaction: $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$ (Equation 2)

Ion exchange membranes may be applied to separate the anodic and cathodic reactions (referred to two-chamber MSC). To electro-neutralize the positive charge at the anode, protons (or other cations) move from anode to cathode. Anodic reactions produce protons and in contrast cathodic reactions consume protons (see Equation 1, 2 and Figure 2.2). In correlation to this occurrence, the presence of ion exchange membranes (typical in MFCs) cause pH decrease in anodic liquid and pH increase in cathodic liquid (pH gradient). They are due to the fact that migration of protons from anodic chamber to cathodic chamber is slower than the proton production rate in the anode chamber and the proton consumption rate in the cathode chamber (Rozendal et al. 2006). Thus, ions other than protons migrate across membranes to maintain charge balance. The pH gradient may result in a low current generation due to the decreased voltage and inhibition to biocatalyst life-cycle. Membrane-less configurations (referred to single chamber) have been tested in MFCs with the power production was reported double compared to the two chambers (Liu et al. 2004). In MSC, membrane-less configuration was used to indirectly convert solar energy into electricity through in situ oxidation of photosynthetically generated hydrogen (Cho et al. 2008).

MSC has potential application for power production in combination to the production of valuable compounds (McCormick et al. 2015), removal of micro-nutrient *e.g.* phosphate and nitrogen from effluent of waste water treatment (Gonzalez del Campo et al. 2015; Zhang et al. 2011) as well as biosensing. Since electrons source is water, electron donor supply is not required in MSCs, making them suitable for application in a remote location (Cao et al. 2008).

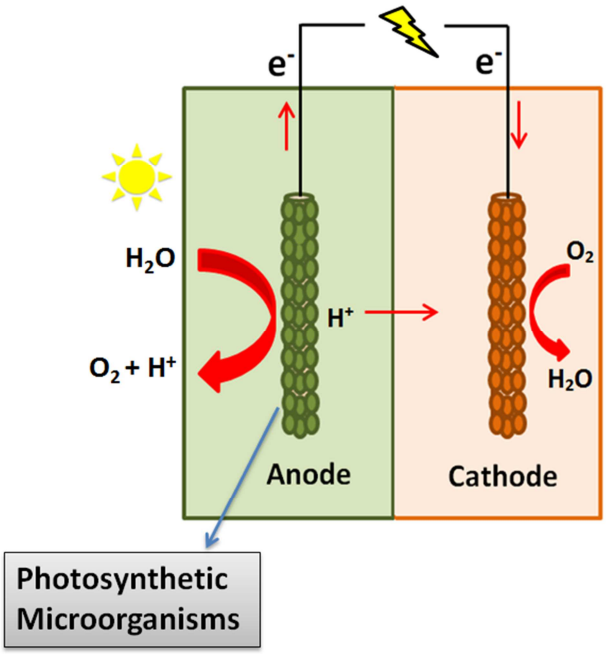

Figure 2.2 Schematic representation of a MSC

2.3 Occurrence of photosynthetic microorganisms

Photosynthesis is the fundamental metabolic processes in photosynthetic organisms, which only need water, atmospheric CO_2, minerals and are driven by solar light (Mur et al. 1999). Two types of photosynthetic microorganisms – cyanobacteria and microalgae – were widely studied for their catalytic capability to generate power in MSCs.

In the case of cyanobacterium *Synechocystis sp.* PCC 6803, photosystems (PS2 and PS1), PETC and respiratory electron transport chain (RETC) are located in thylakoid membranes (Liberton et al. 2006) and both share electron carriers such as PQ, Cyt *b6f* and PC (Scherer 1990) (Figure 2.3). A pair of thylakoid membranes (separated by thylakoid lumen) forms a thylakoid and is floating in cytoplasm (Liberton et al. 2006). In contrast to microalgae (eukaryote), the thylakoid membranes in cyanobacteria (prokaryote) are only separated from the outer world by a relatively smaller quantity of membranes (Bryant 1994) (Figure 2.3). With fewer cell membranes, cyanobacteria are thought to conduct EET more easily than microalgae (Freguia et al. 2012). Moreover, the ability of cyanobacteria to transfer electrons via PETC in light and RETC in dark will be potentially exploited to generate electrical power continuously during both day and night.

Moreover, cyanobacteria use chlorophyll-*a* as a major pigment and almost all cyanobacteria possess other pigments such as phycobiliproteins (allophycocyanin,

phycocyanin and phycoerythrin) for harvesting light and conducting photosynthesis (Herrero and Flores 2008). All the phycobiliproteins form phycobilisomes, antennae structures of the light-harvesting located on the thylakoids membranes (Stal 2007) (Figure 2.3). The majority of cyanobacteria use both chlorophyll-*a* and phycobiliproteins pigments more easily (compared to microalgae), and therefore they are able to harvest solar light more efficiently for energy metabolism (Mur et al. 1999). These facts provide more potential for cyanobacteria to function as biocatalyst and to conduct EET. The different characteristics between cyanobacteria and microalgae may influence the EET features of natural microbial consortia in MSCs.

In most studies done with pure cultures of photosynthetic microorganisms, higher photo-anodic currents were observed during illumination than in darkness (Tanaka et al. 1988; Yagishita et al. 1999; Pisciotta et al. 2010). Interestingly, the opposite profile was observed in the presence of photosynthetic consortia (He et al. 2009). These occurrences may involve the contribution of heterotrophs in consortia – leading to a higher kinetic rate of respiration on organic matter during the dark. Instead, cathodic current was reported during illumination of photosynthetic consortia (He et al. 2009; Strik et al. 2010), leading to a phenomenon known as reversible current, where the current is anodic during night time and cathodic during day time. So far, no reversible current was reported for pure cultures of photosynthetic microorganisms. It was proposed that the cathodic current generation is correlated with the presence of bacteria which possess enzymatic machinery for oxygen reduction, *e.g.* gamma-proteobacteria: *Congregibacter* (Rothballer et al. 2015), *Marinobacter* spp. (Strycharz-Glaven et al. 2013) and green non-sulfur bacteria (Chloroflexi) for the case in MFC (Blanchet et al. 2014). The different current profiles observed with different cultures point to the paramount role of functionally different communities in MSCs. Therefore, understanding how community composition in photosynthetic consortia affects the EET capabilities is very useful to enable a better selection of biocatalyst.

A low photo-anodic current catalysed by photosynthetic microorganisms in MSC has been postulated as being affected by photosynthetic evolved O_2 that compete with the anode as final electron acceptor (Girguis et al. 2010; Strik et al. 2011). A similar effect was reported in MFCs, when O_2 was found to be reduced by electrons excreted by heterotrophic bacteria, causing detrimental effect on anodic current generation (Harnisch and Schroeder 2009; Pham et al. 2004). Moreover, the observed cathodic current was proposed due to

enzymatic oxygen reduction. Unfortunately, the full extent of these O_2 effects on the photo current outputs in MSCs has not been well studied (McCormick et al. 2015; Chen et al. 2014) and needs to be thoroughly investigated for a better design of MSC device.

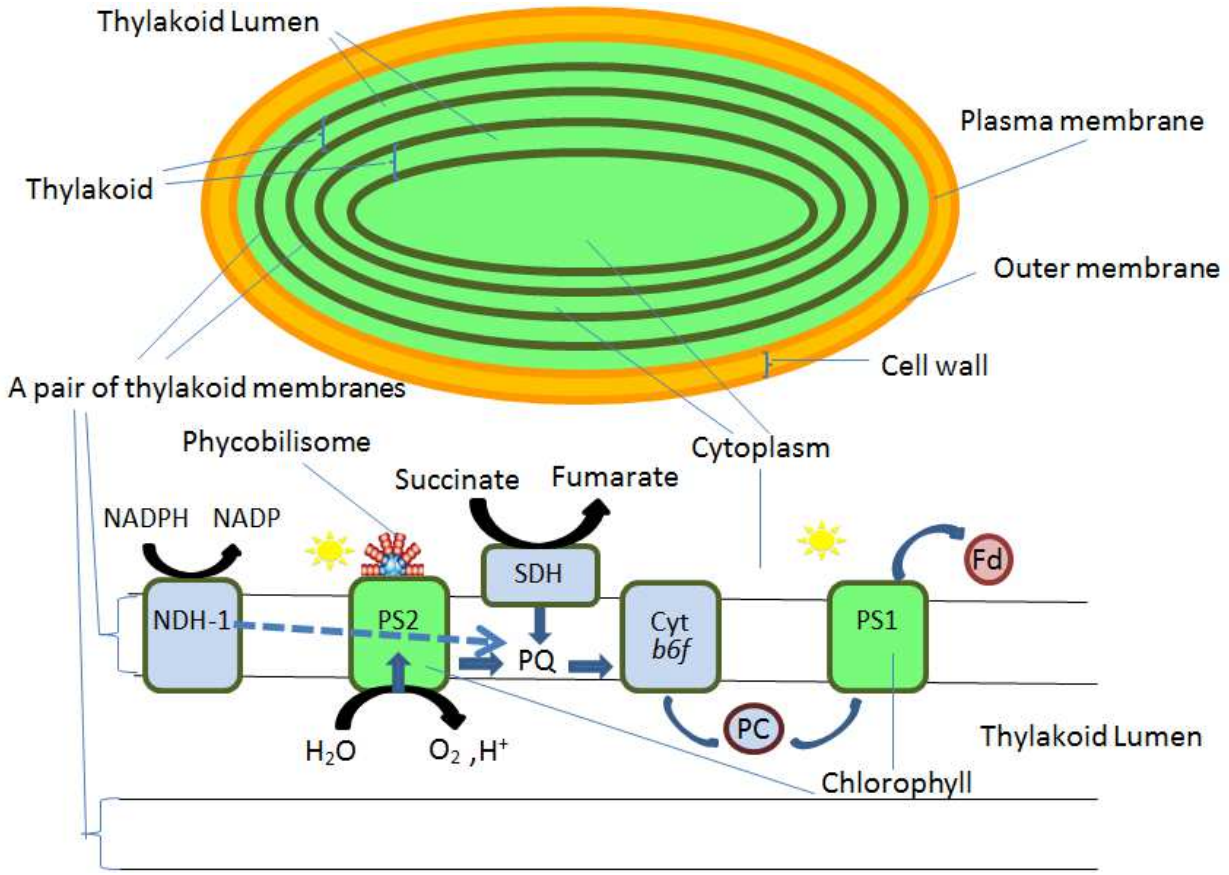

Figure 2.3 Schematic representation of a thylakoid membrane with associated PETC and RETC (respiratory electron transfer chain) in *Synechocystis* sp. PCC 6803. Blue arrows show a flow of electrons

2.4 EET pathways and metabolisms in cyanobacteria

Some studies have revealed that electrons are able to migrate from the cyanobacterial photosynthetic electron transport chain to insoluble extracellular electron acceptors. Pisciotta et al. (2011) used site-specific inhibitors to target enzymes and co-factors in PETC of cyanobacteria (*Nostoc* and *Lyngbya*) and discovered that electrons were extracellularly transmitted from PS2 via PQ components. It was also reported that electrons leave the PETC at the reductive side of PS1 – Fd – in cases of *Synechocystis sp.* PCC 6803 (McCormick et al. 2011; Bombelli et al. 2011) and *Synechococcus* sp. (Yagishita et al. 1993). How the electrons delivered from outer side of cells to electrode and what possible electron sources involved are illustrated in Figure 2.4.

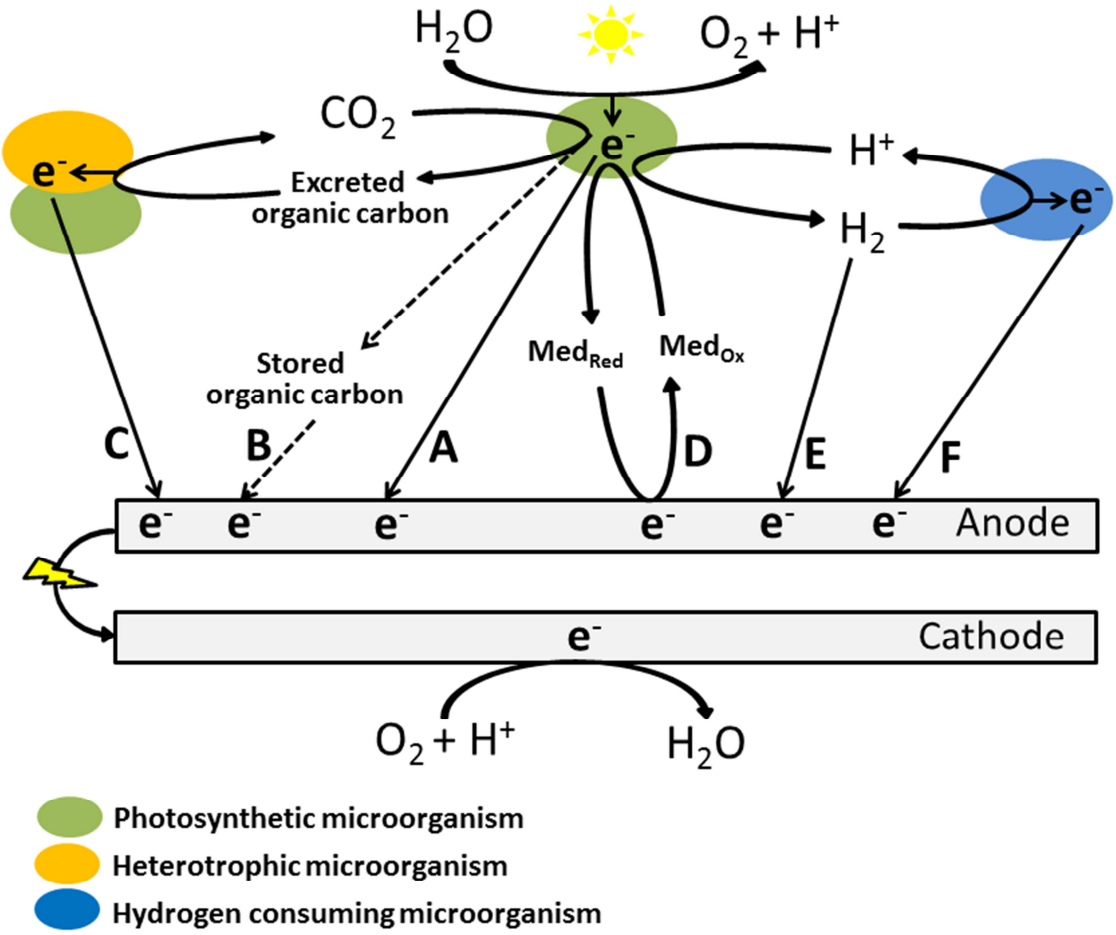

Figure 2.4 Illustration of solar energy conversion to electricity (A) directly from water splitting; or indirectly via respiration of (B) stored and (C) excreted photosynthetic organic substrate and/or cell debris, (D) cycling of excreted redox mediator, (E) electrochemical oxidation and (F) respiration of photosynthetic evolved hydrogen

Cyanobacteria may possess several EET pathways to insoluble electron acceptors like electrode (see Figure 2.4): (A) directly from photosynthetic microorganism to electrode – known as direct extracellular electron transfer (DEET) (Zou et al. 2009; Pisciotta et al. 2010; Sekar et al. 2014); or indirectly via respiration on (B) stored (Strik et al. 2008; Strik et al. 2010) and (C) excreted photosynthetic organic substrate (Chaudhuri and Lovley 2003) and/or cell debris (Madiraju et al. 2012); (D) cycling of excreted redox mediator (McCormick et al. 2015); (E) electrochemical oxidation (Rosenbaum et al. 2005; Cho et al. 2008) and (F) respiration on photosynthetic evolved hydrogen (Olson and Maier 2002).

As illustrated in Figure 2.5, DEET in cyanobacteria may hypothetically occur via two mechanisms: (i) through contact between microbial cell (via cytochromes) and electrode (see mechanism A), as suggested for *Leptolyngbya* (Zou et al. 2009; Pisciotta et al. 2010)

and reported for *Nostoc* sp. (Sekar et al. 2014), (ii) through contact via electrically conductive nanowire (see mechanism B), as observed in *Synechocystis* PCC6803 (Gorby et al. 2006).

The harvesting of solar light via photosynthetic intermediate metabolites (see pathway B, C, E and F in Figure 2.4) is called indirect extracellular electron transfer (IEET). In MSC, organic matter that may be available outside the cell, excreted by phototrophs (Chaudhuri and Lovley 2003) and/or in form of the degraded cell debris (Madiraju et al. 2012) – can provide organic substrate for heterotrophic respiration, as described in equation 3.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad \text{(Equation 3)}$$

Some cyanobacterial species are found to live in the complete absence of light. They possess ability to store metabolites within their cytoplasm such as glycogen (the main reserve substrate), and then use it as energy source over respiration to survive in darkness regions (Herrero and Flores 2008). It was observed that while cyanobacteria colonies sink to the bottom of the water body, they gradually consume their metabolites storage by respiration (Mur et al. 1999). This natural occurrence was also witnessed in MSC devices catalysed by single species of cyanobacteria. Tanaka et al. (1985) reported that current was produced in the dark by cyanobacteria *Anabaena variabilis* through RETC over oxidation of stored glycogen. Yagishita et al. (1993) also showed a direct relationship between the stored glycogen in *Synechococcus sp*. and the current generated during light/dark cycles.

It is known that glucose-6-phosphate dehydrogenase in cyanobacteria is inactivated by light (Mann 2002; Stanier and Cohen-Bazire 1977; Tanaka et al. 1985), therefore eliminating the possibility of metabolism via respiration on stored organic carbon under illumination.

Another possible IEET pathway to electrode is via excreted redox mediator by microorganisms (see pathway D in Figure 2.4). It was reported that *Pseudomonas aeruginosa* excretes redox shuttle called phenazines (Venkataraman et al. 2011), *Shewanella oneidensis MR-1* excretes both a quinone-like molecule (menaquinone) (Newman and Kolter 2000) and riboflavin (von Canstein et al. 2008). However, to our

knowledge no photosynthetic microorganisms have been reported to possess such pathway.

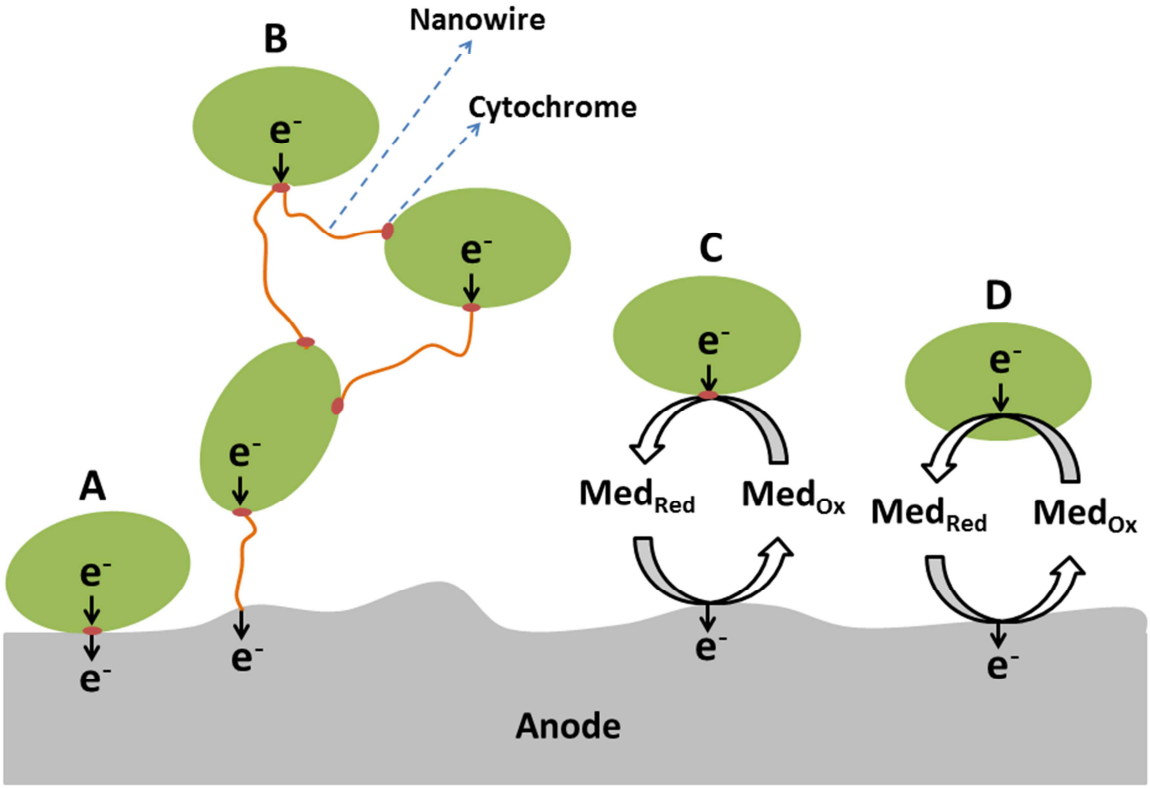

Figure 2.5 Model of EET mechanisms in photosynthetic microorganisms: DEET via (A) cytochrome and (B) nanowire; IEET via cycling of (C) added mediated between outer membrane cell – cytochrome – and anode, (D) added or excreted redox mediator between inner membrane cell – electron carrier in PETC – and anode

Many studies on solar energy conversion into electricity by pure cultures of cyanobacteria have been conducted: without redox assistance of electron shuttling mediators (Bombelli et al. 2011; Lin et al. 2013; Pisciotta et al. 2010; Sekar et al. 2014), or with mediators (Tanaka et al. 1985; Tsujimura et al. 2001; Yagishita et al. 1997). However, only a limited number of reports can be found on environmental phototrophic mixed cultures (Zou et al. 2009; Pisciotta et al. 2010) (see Table 2.1). The observed electric current for the latter case may involve the combination of DEET and IEET (McCormick et al. 2015). The complete pathway of EET from photosynthetic consortia to electrode and their metabolism remain unclear, being the most important knowledge gap to examine.

Photosynthetic microorganisms are known to dissipate the excess energy as heat to protect their photosystems from damage under stressing condition *e.g.* high light intensity (Horton et al. 1996; Müller et al. 2001). In cyanobacteria, the excess energy is driven away through several mechanisms, such as quenching of excited state phycobilisomes (using orange carotenoid proteins (El Bissati et al. 2000; Kirilovsky 2007; Rakhimberdieva et al. 2010), oxygen photoreduction to water in PS1 (Badger et al. 1985) and PS2 (Shimakawa et al. 2015), photoprotection against reactive oxygen species (ROS) by ROS-scavenging enzymes in PS2 (Aro et al. 1993; Zhang et al. 2009) and hydrogen formation by N_2-fixing enzyme nitrogenase (Igarashi and Seefeldt 2003). EET activities on photosynthetic microorganisms are also suggested as a way for disposal of excess electrons (Cereda et al. 2014; Pisciotta et al. 2011; Davey et al. 2003). To achieve long-term application, however, more information is required on the reasons behind this EET feature.

Table 2.1 Current densities (normalized to anode surface) produced in MSC with cyanobacteria as biocatalyst

Cyanobacteria	Mediator	light intensity (Wm^{-2})	Current density ($mA\ m^{-2}$)	Reference
Synechococcus sp.	2-Hydroxy-1,4-naphthoquinone	40	3000 (uncontrolled)	Yagishita et al., 1997
Synechococcus sp. PCC7942	2,6-Dimethyl-1,4-benzoquinone	15	8000 (potentiostatically)	Tsujimura et al., 2001
Nostoc sp. ATCC 27893	None	760	250 (potentiostatically)	Sekar et al., 2014
Pond cyanobacterial mixed cultures	None	0.15	100 (uncontrolled)	Pisciotta et al., 2010

2.5 Electron shuttling redox mediator

In order to enhance flux of electrons from the photosynthetic microbial cells to electrodes, mobile redox components have been externally added. Principally, the mobile redox mediator becomes reduced while taking up electrons from inner or outer membrane of the cell (see profile C and D in Figure 2.5, respectively), shuttles the electrons to the electrode and are oxidised, then cycles back in its oxidised forms to the microbial cell (Carmona-

Martinez et al. 2011). Mediators 2-hydroxy-1,4-naphtoquinone (HNQ) (Tanaka et al. 1988; Tanaka et al. 1985; Yagishita et al. 1999; Yagishita et al. 1998) and 2,6-dimethyl-1,4-benzoquinone (DMBQ) or diaminodurene (DAD) (Tsujimura et al. 2001) have been tested and they effectively transported electrons from cyanobacterial cultures to the anode.

The use of mobile mediators is regarded as unsustainable, expensive in term of continuous replacement and in some cases even harmful to the natural environment (Rosenbaum et al. 2010). More disadvantages are related to the necessity of ion exchange membrane used to prevent anodic/cathodic crossover reactions of the mediator (Kavanagh and Leech 2013), and the negative effect to microbe viability (Martens and Hall 1994). To overcome these weaknesses, some works have been conducted and efficiently improved electron fluxes from microorganisms to anodes through immobilisation of polymeric mediators onto anodes, such as polymeric osmium complexes (Osadebe and Leech 2014) and polymeric azines (*e.g.* polymethylene blue (Marinho et al. 2012) , polythionine (Bauldreay and Archer 1983) and polymethylene green (Yang et al. 1998). Importantly, Hasan et al. (2014) observed an improved biocatalytic performance of cyanobacterium *Leptolyngbia* with polymeric osmium complexes-immobilised anode in MSCs. Application of the immobilisation technic in MSCs containing environmental photosynthetic consortia is of importance, not only to advance the EET capability, but also to enable an economically feasible design of the device. Therefore, more experimental work is required to test redox polymer immobilisation in mixed culture MSCs.

2.6 Research objectives, hypotheses and outcomes

As a novel technology, more fundamental aspects of the process need to be addressed before considering the scaling-up and commercial application of MSCs. Based on the current literature and the identified knowledge gaps, the objectives are herewith pointed out. Hypotheses and the summary of outcomes are also described.

Objective 1: *Investigating whether the capacity of EET is common in environmental phototrophic cultures*

In this objective, we elucidate the natural occurrence of EET capacity in environmental phototrophic cultures and whether the microbial community compositions affect the generation of current. We aim to expand the literature work by investigating a number of environmental samples including seawater and lake water cultures.

Hypothesis: Based on literature data, we hypothesize that environmental phototrophic EET is common and the presence of different microorganisms in the consortia affects the electric performance.

Outcome: Five fresh water and three seawater cultures were enriched from different lakes and shores prior to use as inocula. We found that EET was not active in any of the fresh water cultures used in this study. There was only minimal electron flux in the presence of an added soluble mediator (riboflavin). Biomass identification and fluorescence in situ hybridisation (FISH) conducted in two enriched fresh water cultures showed that the majority of microorganisms was microalgae in both consortia. Details from one culture are shown in **Chapter 3**. Contrarily to freshwater systems, EET seems to be ubiquitous in seawater environments with two tested enriched biofilms shown to be dominated by cyanobacteria (see **Chapter 4)**. The EET capabilities might be attributed to the superior electron transfer characteristics of cyanobacteria over eukaryotic microalgae in both seawater consortia. Cyanobacteria, with fewer cell membranes are thought to be able to conduct EET more easily than microalgae. Moreover, within an electrochemically active seawater culture we found a link between the existence of a dual microbial community – phototrophs (cyanobacteria) and oxygen reducing bacteria (gamma-proteobacteria) – and the generation of fully reversible current: cathodic during the illumination and anodic at dark time. With the absence of gamma-proteobacteria, the consortium exhibited negligible daytime output but high anodic current at night. These occurrences indicate that microbial community composition strongly affects the nature and extent of EET.

Objective 2: *Elucidating the link between photosynthetic oxygen and the observed phenomenon of reversible currents*

In this objective, the observed reversible current was studied in more detail for its correlation with photosynthetic evolved oxygen.

Hypothesis: In MFCs, oxygen is found to oxidise the reduced electron carriers excreted by electrogenic bacteria causing detrimental effect on anodic current generation. The cathodic current observed under illumination in MSCs was also linked to the effect of oxygen. It is therefore expected that photosynthetically produced oxygen plays a critical role on EET in phototrophic consortia.

Outcome: Photosynthetic oxygen is detrimental to anodic processes in fresh and seawater environments. As shown in **Chapter 3**, oxygen re-oxidised the reduced form of riboflavin – leading to a lower anodic current production during the illumination than dark time. In **Chapter 4**, it is revealed that oxygen reduction was responsible for generation of day time-cathodic current (instead of anodic current).

Objective 3: *Elucidating the metabolic reasons for the occurrence of EET in mixed phototrophic communities*

This objective aims to develop an understanding of the evolutionary reasons behind the EET feature of photosynthetic life, in particular to elucidate the metabolism and pathways of phototrophic consortia's EET. For this and the following objectives, the most EET-active consortium dominated by seawater cyanobacteria was used (as characterised as part of Objective 1).

Hypothesis: Hydrogen, stored and excreted organic matter as well as cell debris are known as cyanobacterial photosynthetic products and all could be primary electron donors for EET in these systems. It is also possible that EET is carried directly by phototrophs as a way to dispose of excess electrons under reductive stress. It is expected that EET mechanisms in environmental cyanobacterial samples are dominated by IEET due to the long path of electron travel between the photosynthetic reaction centres and the extracellular environment. Besides PETC, we expect that respiration also play a role in electricity generation, especially during night time.

Outcome: The flux of electrons under illumination was generated by photosynthetic bacteria – mostly cyanobacteria – as a direct effect of light, via water splitting and possibly intermediate electron carriers such as hydrogen. The exogenous accumulation of organic matter was found to not affect the day and night time-EET. During the dark, electron flux is correlated to the extent of photosynthesis during the previous day – storage of fixed CO_2 in day time. Also, the electron flux onto electrodes may be a natural way for cyanobacteria and other phototrophic microorganisms to maintain redox balance for survival under reductive stress arisen from low inorganic carbon concentrations or high light intensity. This work points towards physiological and metabolic reasons for the observed phenomenon. A complete explanation of this outcome is detailed in **Chapter 5**.

Objective 4: *Enhancing performance towards creation of MSC devices.*

For this objective, the influence of immobilisation of two different polymeric osmium complexes and three polymeric azine mediators (polymethylene blue and green, polythionine) onto working electrodes on the extent of electrogenic activity of environmental phototrophic cultures is determined.

Hypothesis: It is known that immobilisation of polymeric osmium complexes and polymeric azine mediators onto working electrodes improves MFC performance. Polymeric osmium complexes have been also successfully tested in MSC catalysed by single species of cyanobacteria and microalgae. Therefore, the immobilisation of polymeric mediators is expected to efficiently shuttle electrons between cyanobacterial consortial biofilm and electrodes and thus lead to higher current densities.

Outcome: The maximum improvement of anodic current outputs were achieved with electrodes immobilised by polymeric osmium complex [Os(2,2'-bipyridine)$_2$(polyvinyl-imidazole)$_{10}$Cl]Cl, from 6-9 mA m^{-2} in night time and 5 mA m^{-2} during the day with bare graphite electrode to 317 ± 29 mA m^{-2} (43 ± 8-fold) and 320 ± 28 mA m^{-2} (64 ± 6-fold), respectively. Interestingly, the detrimental effect of oxygen to photo current generation was not observed in the presence of immobilised polymers: higher day time-anodic current was exhibited than at night. This outcome is detailed in **Chapter 6**.

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Chapter 3

Oxygen suppresses light-driven anodic current generation by a mixed phototrophic culture

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This chapter describes the detrimental effect of photosynthetically evolved oxygen on anodic current generation in the presence of riboflavin upon illumination of a mixed phototrophic culture enriched from a freshwater pond at +0.6 V vs standard hydrogen electrode. In the presence of riboflavin, the phototrophic biomass in the anodic compartment produced an electrical current in response to light/dark cycles (12 h/12 h) over 12 months of operation, generating a maximum current density of 17.5 mA m^{-2} during the dark phase, whereas a much lower current of approximately 2 mA m^{-2} was generated during illumination. We found that the low current generation under light exposure was caused by high rates of re-oxidation of reduced riboflavin by oxygen produced during photosynthesis. Quantification of biomass by fluorescence in situ hybridization images suggested that green algae were predominant in both the anode-based biofilm (55.1%) and the anolyte suspension (87.9%) with the remaining biovolume accounted for by bacteria. Genus-level sequencing analysis revealed that bacteria were dominated by cyanobacterium *Leptolyngbia* (~35%), while the prevailing algae were *Dictyosphaerium, Coelastrum*, and *Auxenochlorella*. This study offers a key comprehension of mediator sensitivity to reoxidation by dissolved oxygen for improvement of microbial solar cell performance.

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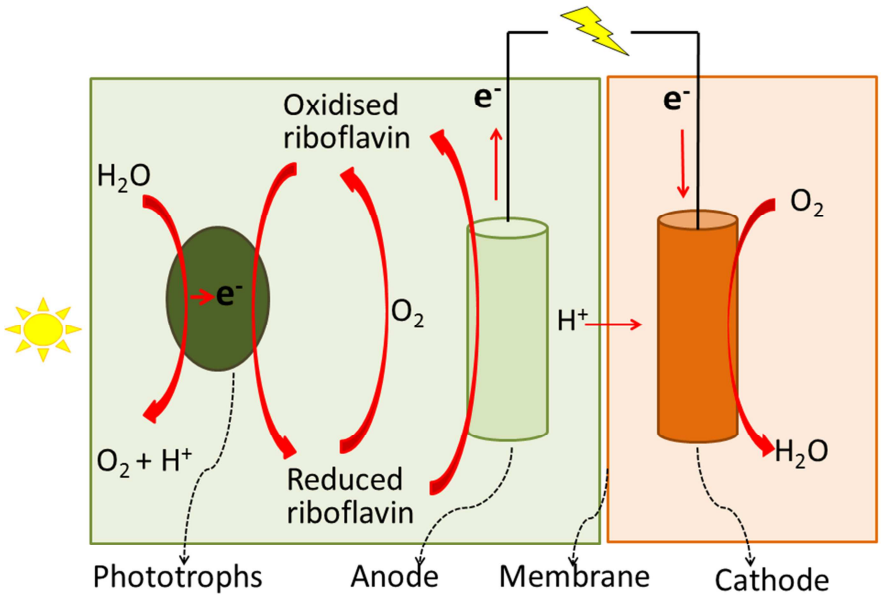


3.1 Introduction

Due to the global issues of fossil fuel depletion and climate change, the development of renewable solar energy-harvesting technologies has gained momentum. Microbial solar cells (MSCs) are an innovative type of bioelectrochemical system (BES), able to turn solar energy into electricity through photosynthetic activity within a fuel cell-type encasing (Freguia et al. 2012; Strik et al. 2011; Rosenbaum et al. 2010). During photosynthesis in the anodic chamber of a MSC, the light-driven reaction splits water into oxygen, protons and electrons, while CO_2 is concomitantly fixed endogenously into glycogen and new cell material, or protons are reduced into hydrogen by phototrophic microorganisms (Benemann 1997). The electrons produced from water splitting (Zou et al. 2009; Pisciotta et al. 2010) and oxidation of intermediate metabolites such as stored glycogen (Tanaka et al. 1985; Tanaka et al. 1988) and hydrogen (Cho et al. 2008; Rosenbaum et al. 2005) are able to generate an electric current in light and in dark conditions, respectively. The electrons thus generated are used for the reduction of oxygen (or other compounds) in a cathodic chamber (Strik et al. 2010).

MSC anodes containing pure and mixed phototrophic cultures have been successfully tested with and without mediators (added to facilitate electron transfer from the cultures to the anode). Several pure cultures of cyanobacteria have been found to respond electrically in MSCs without the use of mediators, for example, *Synechocystis spp*. (approximately 1.3 mA m^{-2}) (Madiraju et al. 2012) and *Synechococcus spp.* (approximately 93 mA m^{-2})

(McCormick et al. 2011), but current production has been lower than observed for other BESs. Conversely, the addition of exogenous mediators has resulted in a significant increase in current output, as is the case for *Anabaena spp*. (approximately 4.5 A m^{-2}) (Tanaka et al. 1988) and *Synechococcus* spp. (approximately 400 mA m^{-2}) (Yagishita et al. 1993) in the presence of 2-hydroxy-1,4-naphthoquinone (HNQ). These investigations usually showed higher current production during the light phase than in the dark. In some cases, however, lower current output was observed during the light phase when mixed phototrophic cultures were employed. He et al. (2009) suggested that this effect was due to current generation inhibition caused by photosynthetically evolved oxygen (which can be measured in the form of dissolved oxygen, DO) produced by photosynthesis during the light phase. It is known that DO reduction by biologically derived electrons within the anode chamber of a microbial fuel cell (MFC) prevents extracellular electron transfer to the anode, thus hindering electricity generation (Harnisch and Schroeder 2009), but this effect has not been thoroughly investigated in MSCs.

In this work, we studied the current generation in the presence of riboflavin with a mixed phototrophic bioanode culture as biocatalyst. The use of riboflavin is interesting because it is naturally produced and utilized by bacteria to mediate extracellular electron transfer in biogeochemical metal cycling (Marsili et al. 2008) and in MFC anodes (Li et al. 2012). The self-mediated electron transfer from bacterial cells to MFC anode in the presence of natural consortia was also reported (Rabaey et al. 2004), hence providing an opportunity for a possible synergistic cooperation between chemotrophs and phototrophs in MSCs. This work demonstrates the detrimental effect of oxygen on current generation by a mixed phototrophic culture in the presence of riboflavin during illumination.

3.2 Experimental section

3.2.1 Microbial solar cells setup

The experiments were conducted in a dark box and used two types of two-chamber photoreactors, flat-plate type and bottle type (described below), illuminated by cool white fluorescent lamps (Pisciotta et al. 2010) at the side facing the anode chambers. All experiments were performed in duplicate using two identical reactors. Unless otherwise specified, a 12 h light and 12 h dark phase was maintained by a time-switch controller at 135 μmol m^{-2} s^{-1} light intensity (measured at inner wall of reactors). In order to prevent temperature increase inside the dark box, the box was fitted with two ventilation fans which were operated continuously and hence maintained room temperature conditions (23−25

°C). A modified Cyanophycean medium (0.05 g L^{-1} K_2HPO_4, 0.05 g L^{-1} $MgSO_4 \cdot 7H_2O$, 4.5 mg L^{-1} $Na_2EDTA \cdot 2H_2O$, 0.582 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 0.246 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.03 mg L^{-1} $ZnCl_2$, 0.012 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 0.024 mg L^{-1} $Na_2MoO_4 \cdot 2H_2O$, and 3.36 g L^{-1} $NaHCO_3$ as a buffering agent) at pH 8.0 was fed continuously to the anodic chambers with a syringe pump to maintain a 16-day hydraulic retention time. The cathode chambers were filled solely with phosphate buffer (6 g L^{-1} Na_2HPO_4 and 3 g L^{-1} KH_2PO_4) at pH 8.0.

The flat-plate photoreactors were fabricated using four transparent Perspex frames with outer and inner dimensions of 13 × 28 × 1 cm and 5 × 20 × 1 cm, respectively. Graphite felt (thickness: 1 mm, Morgan Industrial Carbons, Australia), cation exchange membrane (CEM) (Ultrex CMI-7000, Membrane International) and titanium mesh (mesh size 1 mm × 2 mm, thickness 0.15 mm−Kaian Metal Wire Mesh Co. LTD, China) each with the same surface area of 100 cm^2 were utilized as anode, membrane and cathode materials, respectively (see Supporting Information (SI) Figure 3.S1). The electrolytes in both chambers were continuously circulated using a peristaltic pump with a flow rate 2.5 L h^{-1} via a recirculation system equipped with two 250 mL opaque bottles for anolyte and a 250 mL transparent bottle for catholyte, resulting in total liquid volumes of 600 and 350 mL for anode and cathode chambers, respectively. In the anolyte circulation system, the first bottle (located after the anodic outlet of the photoreactor) functioned as a sample port and housed the online pH and DO sensors. The second bottle, located immediately downstream, was used for gas sparging, release of produced gases and to draw liquid effluent. All circulation bottles were placed in the dark box. To achieve an efficient mixing, the anolyte was continuously stirred at 500 rpm using magnetic bars (diameter 0.5 cm and length 2 cm) in both circulation bottles.

The bottle photoreactors were set up using modified 250 mL borosilicate bottles with the effective volume of anode and cathode chambers solution being 200 cm^3 and 40 cm^3. Graphite felt (surface area 26 cm^2) was used as anode, directly inserted into the bottle; CEM (4.9 cm^2) was used to separate the anode chamber from a small cathode chamber derived from a glass tube, directly inserted into the bottle; a titanium wire (length 4 cm, diameter 0.5 mm, Advent Research Materials, England) was used as cathode. Platinum wire (length 4 cm, diameter 0.5 mm, Advent Research Materials, U.K.) was used as a cathode in a specific set of experiments as described in the Results and Discussion section. The bottles were modified with ports to facilitate continuous-flow feeding, to draw liquid effluent, to enable sparging and releasing of gases, and to enable sampling and

insertion of pH and DO probes. The use of this bottle type reactor had different aims from the flat plate type reactor and the results are not comparable to each other.

For both reactor types, an Ag/AgCl reference electrode (sat. KCl, +0.197 V vs standard hydrogen electrode, SHE) was placed in each anode chamber and Titanium mesh was pressed onto the graphite felt anodes for current collection. The pH of the anolyte was controlled via dosing of 1 M HCl solution to maintain a pH of 8.0 ± 0.2, using a pump connected to programmable logic controller (PLC) system to provide optimum condition for a microbial consortium performance as biocatalyst (Gil et al. 2003), to decrease riboflavin photodegradation in the presence of sodium bicarbonate buffer (Ahmad et al. 2014) and to increase solubilization of CO_2 (Sugai-Guérios et al. 2014).

3.2.2 Analytical methods

The current density was calculated by dividing the observed anodic current, as recorded by a multichannel potentiostat (CHI1000B, CH Instruments), by the projected anode surface area. The pH and DO were continuously measured by two benchtop pH meters (mini-CHEM-pH, Australia) and a benchtop DO meter (O2-4100-e, Mettler Toledo, Germany), and recorded through the PLC system. To determine microbial density of cultures in the anolyte, OD_{660} was measured by spectrophotometer (Cary50 UV−vis, Varian, Australia). The light intensity was measured in W m^{-2} using an IR light sensor (PS-2148, PASPort) and converted to μmol m^{-2} s^{-1} unit.

3.2.3 16S-rRNA gene pyrosequencing, fluorescence in situ hybridization and biovolume fraction

In order to identify the stabilized microbial community performing long-term and reproducible electrochemical activities, biomass attached to anodes as biofilm as well as the suspended cultures in the anolyte of one flat plate reactor were collected 12 months after inoculation by scraping the whole anode surface and centrifuging 200 mL of anolyte. Twenty-five mg of either the biofilm or centrifuged suspended culture were used for 16S-rRNA gene pyrosequence analysis and FISH.

For pyrosequencing, DNA of both samples was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). Extracted DNA was measured for quantity using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technology, Rockland, DE) and quality using agarose gel (1.0%, w/v electrophoresis). Each extracted DNA was sent to the

Australian Centre for Ecogenomics (ACE) at the University of Queensland for 16S-rRNA gene pyrosequencing analysis, using universal primers 926f (5′-AAACTYAAAKGAATTGACGG-3′) and 1392r (5′-ACGGGCGGTGTGTAC-3′) (Amann et al. 1995) with a 454 GS FLX sequencer (Roche, Switzerland), targeting the 16S-rRNA gene regions of the whole community. Sequencing results were analyzed through a local implementation of the ACE Pyrosequencing Pipeline (https://github.com/Ecogenomics/APP). Principally, sequences were demultiplexed and quality filtered by the split_library.py script in QIIME v1.8.0 (Caporaso et al. 2010b). Sequences shorter than 250bp were removed. Sequences were denoised as well as error-corrected with Acacia (Bragg et al. 2012). Each sequence was aligned by PyNAST (Caporaso et al. 2010a) and assigned to operational taxonomic units (OTUs) using UCLUST (Edgar 2010) based on 97% similarity. Taxonomy classifications were then assigned to each OTU by BLAST (Altschul et al. 1990). OTUs identified as chloroplast were filtered out.

Moreover, the FISH was conducted as described in Amann et al. (1995) with hybridization buffer containing 35% of formamide. EUB mix-cy3 probe containing Eub338 (5′-GCTGCCTCCCGTAGGAGT-3′), Eub338II (5′-GCAGCCACCCGTAGGTGT-3′) and Eub338III (5′-GCTGCCACCCGTAGG TGT-3′) for most Bacteria (Amann et al. 1990; Daims et al. 1999) and fluorescing stain 4′6-diamidino-2-phenylindole (DAPI) for total microbial cells (Porter and Feig 1980) were used in this study. Visualization of FISH slides was performed with a Zeiss LSM 510 Meta confocal laser-scanning microscope (CLSM) using two excitation channels (545 nm-red emission and 450 nm-blue emission). Subsequently, 10 randomly chosen images of FISH were quantified by Daime software (Daims and Wagner 2007) to determine biovolume fraction of bacteria to total microbial cells. Bouchez et al. (2000) found that evaluating 10−12 images provided enough total biomass for reliable statistics.

3.2.4 MSC experimental procedures

The phototrophic inoculum was collected from a pond (approximately S 27°29′59.47 E 153° 0′58.25) located at the St. Lucia campus of the University of Queensland (Brisbane, Australia) and subsequently pregrown in flasks placed on an orbital shaker at 100 rpm (Thermoline Scientific, Australia) at room temperature under aerobic conditions (without air sparging) in Cyanophycean medium without KNO_3 (i.e., with air as the only nitrogen source) to promote the growth of cyanobacteria. During the pregrowth the cultures were

illuminated from the top as previously described (135 μmol m^{-2} s^{-1} intensity measured at flask walls and with a standard 12/12 h light/dark cycle).

After pregrowth for a month, the green homogeneous cell suspension was centrifuged and washed twice with phosphate buffer (composition detailed above) to be used as inoculum for long-term experiments utilizing 0.5 mM riboflavin in two identical flat-plate photoreactors. The high concentration of riboflavin was used to prevent the concentration becoming limiting. Approximately one year after inoculation in the latter, the cultures were taken from the anolyte, centrifuged, washed twice with phosphate buffer and used as inoculum for subsequent experiments with the two identical bottle photoreactors. In the majority of experiments, the anode potential was poised at +0.6 V vs SHE, the compartment sparged with a 95% N_2/5% CO_2 gas mix (BOC, Australia) as the sole nitrogen source at a rate 0.5 L min^{-1} and 0.3 L min^{-1} for the flat plate and bottle photoreactors, respectively. Before being supplied to anode chambers, the gas mixture was humidified by flowing it through a bottle containing demineralized water to prevent water loss from the anolyte.

3.3 Results and discussions

3.3.1 Anodic current generation in the presence of riboflavin

Two identical flat-plate photoreactors were fed for 12 months with medium containing 0.5 mM riboflavin and poised to +0.6 V vs SHE anodic potential. Both reactors exhibited observable green phototrophic cultures both in biofilm and suspended forms. The OD_{660} measured in the anolyte was stable around 0.4−0.5 over this period. Figure 3.1 represents a current profile over a number of light/dark cycles after 12 months of continuous operation.

As shown in the figure, these cultures presented different electrical response to light and dark phase conditions. Within the first 2 min of the light phase, the anodic currents dropped and reached a low point (approximately 2 mA m^{-2}) during the rest of the light phase. These output levels are below the previously observed in the presence of mediators, such as a maximum of 1 A m^{-2} generated with a *Synechococcus* sp. PCC7942 MSC34 and 4 A m^{-2} generated with *Synechococcus* sp. UTEX238035 using 2,5-dibromo methyl isopropyl-1,4-benzo-quinone (DBMB) and 2-hydroxy-1,4-naphthoquinone (HNQ), respectively, as mediators. This may be attributed to poor affinity of our mixed phototrophic culture for riboflavin. In contrast to the light phase, the current output during the dark

phase increased gradually to a maximum of 17.5 mA m^{-2} and remained constant even during a 36 h-dark phase (see Figure 3.1). Although the culture could not develop further electrochemical activity to improve current generation, a sustainable current generation was observed with the same pattern of electric response to light and dark phases for over 12 months.

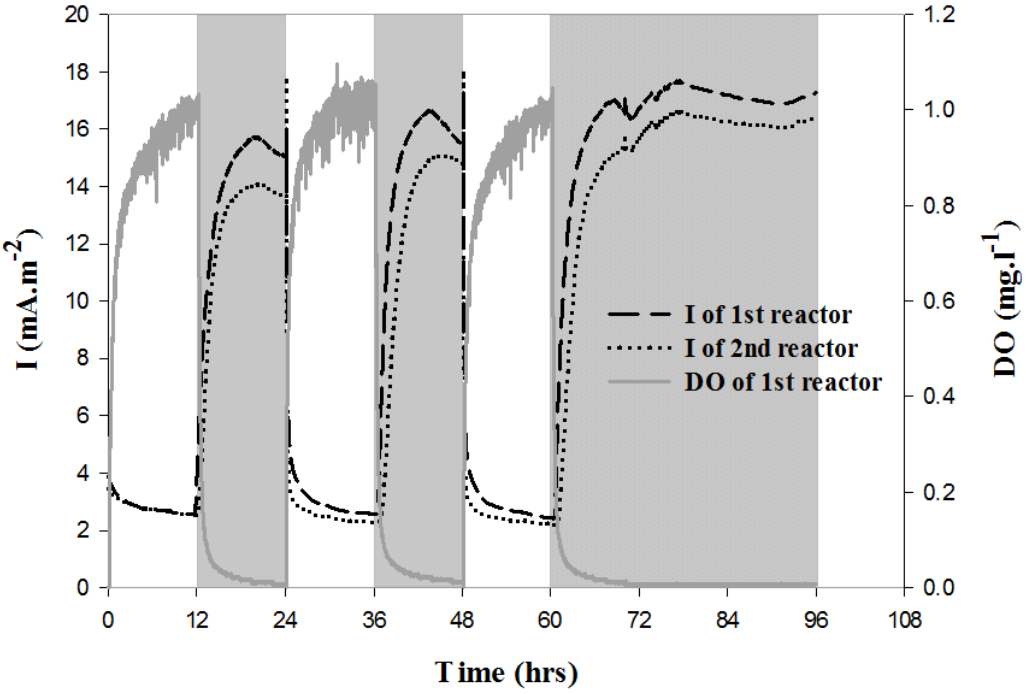


Figure 3.1 Anodic current profile of two identical flat-plate photoreactors (I of 1st and 2nd reactors, E = +0.6 V vs SHE) and DO profile only measured in the 1st reactor, after >12 months exposure to riboflavin; the dark and clear areas symbolize the dark/light phases

To confirm that the current generation was of biological origin and the exogenous riboflavin played a key role in the electron-transfer mechanism within the anodic chamber, a short test was carried using two identical bottle photoreactors in continuous mode. When the latter reactors were run abiotically with Cyanophycean medium and poised at +0.6 V vs SHE for 72 h, no electrical response was observed. Subsequently, an anolyte sample from an operating flat-plate photoreactor was collected, centrifuged, washed twice with phosphate buffer. The pellet was then suspended into a 20 mL medium (OD_{660} 8.6) and inoculated into two bottle photoreactors (10 mL each). Following inoculation, an anodic current developed and reached 17 mA m^{-2} during the dark period (though plummeting to 1 mA m^{-2} when illuminated) within the third day of culture addition (see SI Figure 3.S2). The culture used to inoculate these bottle reactors had been exposed to riboflavin for almost a year, so it is possible that some of the cells carried intracellular or membrane bound

riboflavin, resulting in the observed current. Within the third day of anolyte replacement with medium containing 0.5 mM riboflavin, the current output increased 10 and 2.2-fold, peaking at 10 and 37 mA m^{-2} during light and dark periods, respectively. There was no electrical response in the absence of a phototrophic culture and the current improved significantly following riboflavin addition, revealing that the culture was responsible for the reduction of oxidized riboflavin and that the anodic oxidation of reduced riboflavin had a primary role in the current production of these photoanodes.

3.3.2 Community analysis of biofilm and suspended cells

FISH analyses were conducted to determine the populations of the consortium in both anolyte suspension and anode surface. FISH images of biofilm sample showed the presence of bacteria (see Figure 3.2A), nonbacterial cells (see Figure 3.2B) and the whole community (see Figure 3.2C). Quantification by Daime software showed that the bacteria population occupied 44.9% of the total biovolume fraction in biofilm (100.0 and 10.0% for congruency and standard deviation) and 12.1% of the total microbial biovolume in suspended cells (100.0 and 5.7% for congruency and standard deviation).

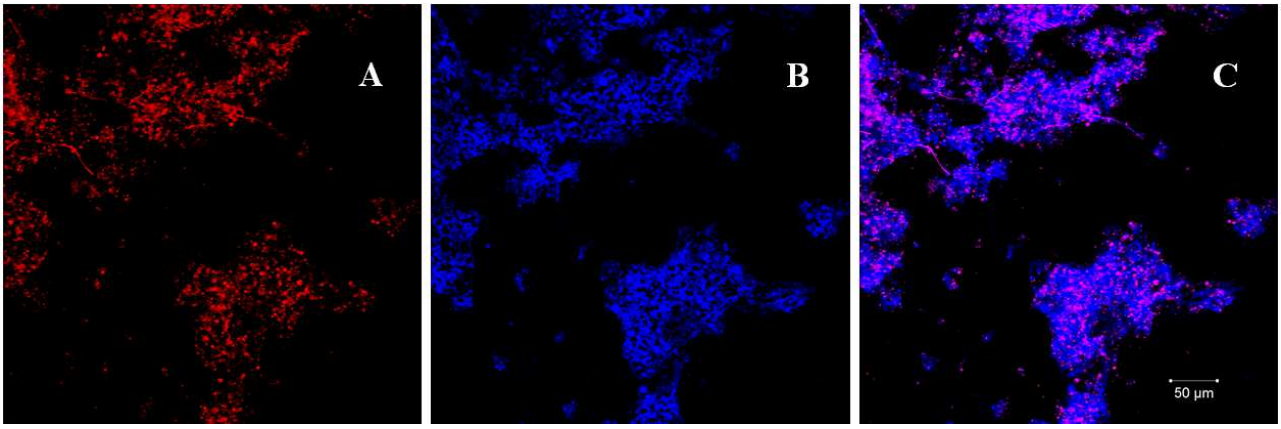


Figure 3.2 FISH images of the microbial community taken from anode surface. (A, B and C) show the same microscopic field, (A) bacteria in red (EUB mix-probe), (B) nonbacterial cells in blue (after subtraction of total cells stained by DAPI with bacterial cells probed by EUB mix), (C) whole community consisting of bacterial cells in pink as a result of overlapping colour of red (EUB mix probe) and blue (DAPI stain) and nonbacterial cells in blue (DAPI), considered to be mainly consisting of microalgae as indicated by sequencing analysis

Sequence analysis of the consortium in both anode surface and anolyte suspension using universal primers confirmed the presence of bacteria and indicated the presence of green

algae. In bacterial community, the prevailing genera were cyanobacterium *Leptolyngbia* accounting for 35.6% in anode surface and 35.0% in anolyte suspension, proteobacteria *Porphyrobacter* (22.9 and 8.9%), *Pseudomonas* (13.5 and 7.8%), *Parvibaculum* (11.9 and 15.2%) and planctomycetes *Rhodopirellula* (16.1 and 33.1%) (see SI Figure S3). At genus level phylogenetically the green algae were most similar to *Dictyosphaerium, Coelastrum*, and *Auxenochlorella*. Considering the presence of *Leptolyngbya* and green algae we speculate that both phototrophs played a relevant role on the anodic electricity production with riboflavin as mediator. Since only N_2 was supplied as nitrogen source, it is reasonable to hypothesize that the growth of green algae was supported by the cyanobacterium *Leptolyngbia* which has the ability to fix N_2, providing a nitrogen source for green algae.

He et al. (2009) hypothesized that photosynthesis in green algae and cyanobacteria supplied organic compounds as electron donors for heterotrophic bacteria in a MSC. Through oxidation of the organic compounds, heterotrophs (proteobacteria and planctomycetes) might also contribute to electricity production in our systems. Proteobacteria are in fact the main drivers of electricity production in MFCs (e.g., *Geobacter* spp. and *Shewanella* spp.). To the best of our knowledge, of the three proteobacteria found in our systems, only Pseudomonas (Rabaey et al. 2005) has been reported to be electroactive. *Porphyrobacter meromictius* is known as an aerobic anoxygenic phototroph, therefore unable to use water as electron source (Rathgeber et al. 2012). Although under anaerobic conditions H_2 is known to be produced by photo/chemoheterotrophic microorganisms (Kapdan and Kargi 2006), no such H_2-producing microorganisms were present in our community.

The affinity of a culture for a particular mediator in a BES depends on how quickly the oxidized form of the mediator can reach the electron transport chain inside the cells and thereby be reduced (Yong et al. 2013). In this particular case, the limited affinity can be attributed to the composition of the phototrophic consortium and the impermeability of cell membranes to riboflavin. The complex membrane of the dominant green algae and the lack of a dedicated riboflavin membrane transporter (Yong et al. 2013) might contribute to a low affinity for riboflavin by limiting the migration transport of oxidized riboflavin to diffusive processes across the cell membrane.

3.3.3 Riboflavin re-oxidation by evolved DO causes lower current production during the light phase

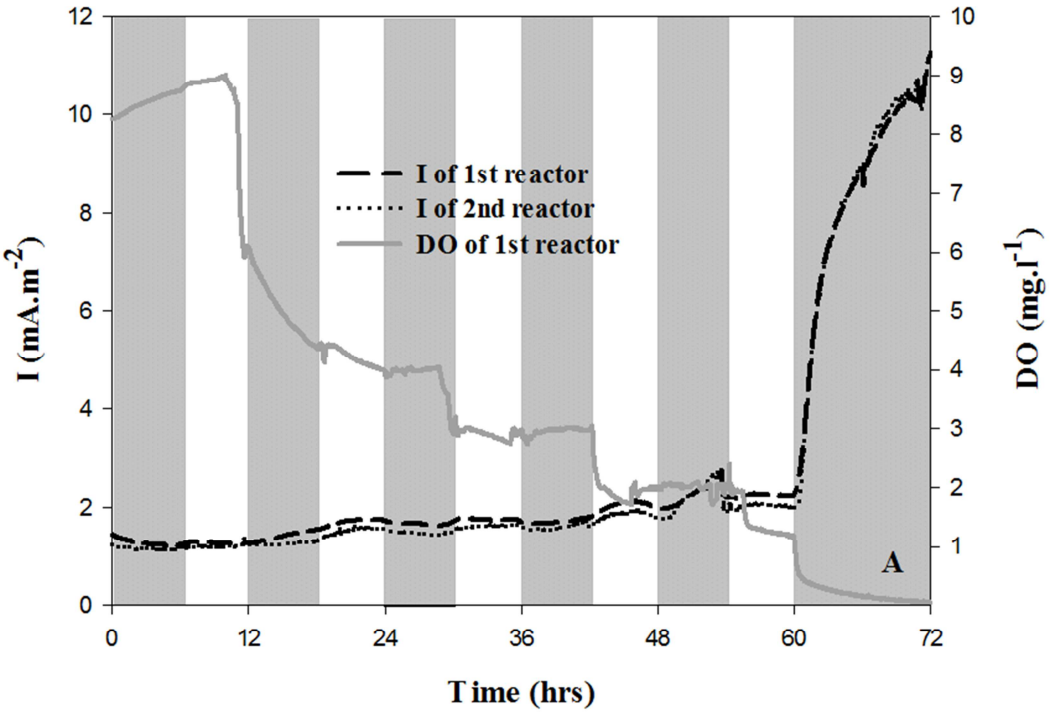
Figure 3.1 illustrates that the DO increases to a maximum of 1 mg L^{-1} during illumination, coinciding with a sudden current drop, and decreases below detection limits during the dark period. In order to experimentally determine whether the current reduction during light exposure was caused by riboflavin reoxidation by evolved DO, a batch test was conducted and repeated twice in a bottle photoreactor (with Pt-wire cathodes) using the same medium as described in the Experimental Section with 0.5 mM riboflavin and without phototrophic cultures. The complete reduction of riboflavin was driven at poised-working electrode value of −0.22 V vs SHE in the absence of DO (continuous sparging with N_2). After the reductive current neared zero, the reaction was changed to oxidation by a potential step to +0.60 V vs SHE, first in the absence of DO, and then followed by the addition of oxygen (by continuous air sparging). During the reduction and oxidation of riboflavin in the absence of DO, the reductive and oxidative currents decreased logarithmically (see SI Figure 3.S4), which is attributable to the depletion of oxidized and reduced forms of riboflavin, respectively. Oxygen exposure during the oxidation of reduced riboflavin resulted in abrupt decrease in the oxidative current to almost zero, suggesting that the oxidation of the reduced riboflavin was entirely carried out by oxygen. This result reveals that the high sensitivity of reduced riboflavin to DO reoxidation strongly contributed to the poor performance of the pond phototrophic culture when exposed to light.

A similar scenario was observed in MSCs by Tanaka et al. (1985) utilizing HNQ as mediator, suggesting that evolved oxygen could be responsible for the reoxidation of the reduced mediator (HNQ) during the light phase. To confirm that dissolved oxygen was the primary reason for the observed inhibition of current generation in our solar anodes during light exposure, three further tests were conducted: (i) control of DO during light/dark phases; (ii) control of DO during an extended dark phase; and (iii) control of light intensity.

As shown in Figure 3.3A, 6 h light/dark cycles were performed and simultaneously the DO was gradually decreased from 9 to 1 mg L^{-1}, resulting in a negligible increase of current only reaching 2 mA m^{-2}. The current production only displayed a substantial increase when the DO concentration was taken down to below 1 mg L^{-1}. This is consistent with the results presented in Figure 3.1, where a DO concentration of 1 mg L^{-1}, due to photosynthetically evolved oxygen, severely limited the current generation during light to around 2 mA m^{-2}.

Due to the continuous oxygen evolution driven by photosynthesis, we were unable to achieve a DO concentration below 1 mg L^{-1} in the DO control test in our reactor setup during the light period. In the following experiment, a gradual increase of DO (starting from zero) was conducted during an extended dark phase. This experiment, presented in Figure 3.3B, shows that an increase of the controlled DO results in gradual decrease of the current. There was no current reduction observed due to depletion of the endogenous stored substrate when the reactors were left under an extended dark phase without DO control for 4 days (see SI Figure 3.S5), indicating that the current drop shown in Figure 3.3B was primarily due to the increase in DO concentration.

Moreover, Figure 3.3C showed that there is a link between light intensity, photosynthetically evolved DO and generated current in the presence of 0.5 mM riboflavin and a poised anode (+0.6 V vs SHE). An increase in light intensity offers more energy for photosynthetic activity and, consequently, more water is split into electrons, protons and oxygen (Lan et al. 2013). As expected, a rapid increase in DO from 0 to 0.3 and then 0.9 mg L^{-1} was observed when the light intensity was increased from 0 to 55 and then to 135 µmol m^{-2} s^{-1}, respectively (see Figure 3.3C). As a consequence, the current dropped with the increase of light intensity.



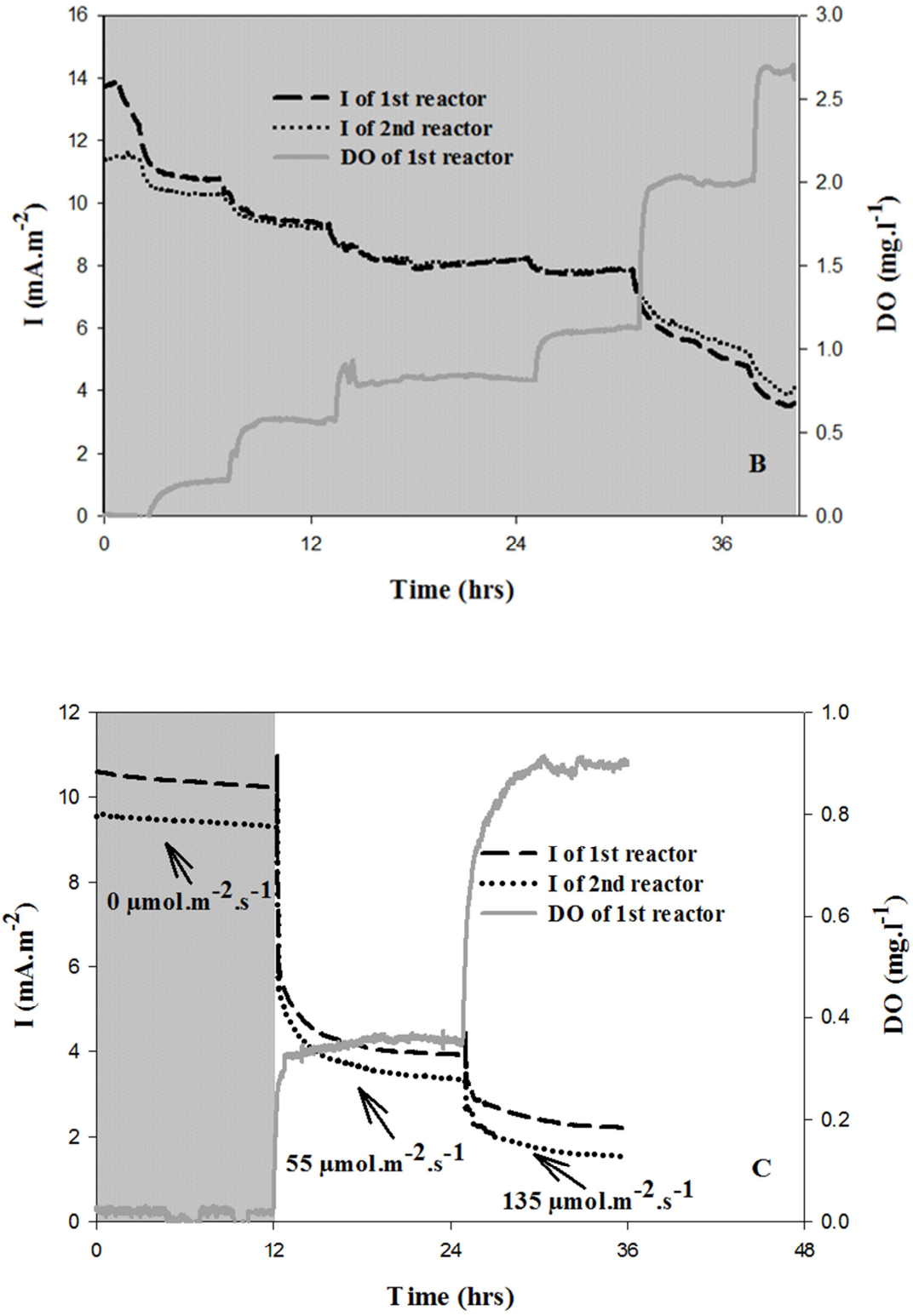


Figure 3.3 Effect of controlled DO (A, B) and light intensity (C) on anodic current profiles (E = +0.6 V vs SHE) of two identical flat plate photoreactors (I of 1st and 2nd reactors) and DO profile (only measured in the 1st reactor) in the presence of 0.5 mM riboflavin. The relatively rough profiles of DO are due to the manual mixing of gases (O_2 and 95% N_2/5% CO_2 mix)

The experimental results demonstrate a univocal link between light, dissolved oxygen and anodic current inhibition in mediated photosynthetic bioelectrochemical processes. The high sensitivity of reduced riboflavin to reoxidation by photosynthetically evolved DO detrimentally affects the anodic current during light exposure. Consideration of mediator sensitivity to reoxidation by DO is essential to make MSC a viable technology. Further investigations are warranted to determine the effect of DO on other mediators and on direct electron transfer pathways.

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3.4.2 Notes

The authors declare no competing financial interest.

3.5 Acknowledgments

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3.6 Associated content

3.6.1 Supporting Information Figures

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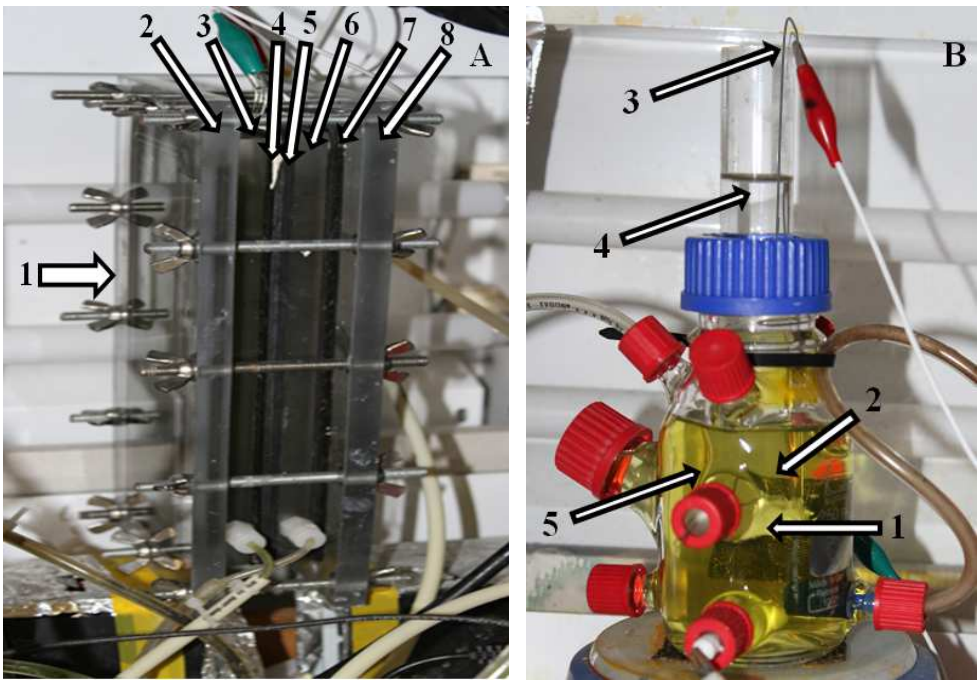

Figure 3.S1 A. Picture of flat-plate photoreactor: (1) light source, (2) front side flat-plate, (3) flat plate, (4) graphite felt as anode, (5) CEM, (6) flat plate, (7) Titanium mesh as cathode, (8) back side flat-plate. B. Picture of bottle photoreactor: (1) CEM, (2) graphite felt as anode, (3) Titanium wire as cathode, (4) cathodic chamber, (5) anodic chamber

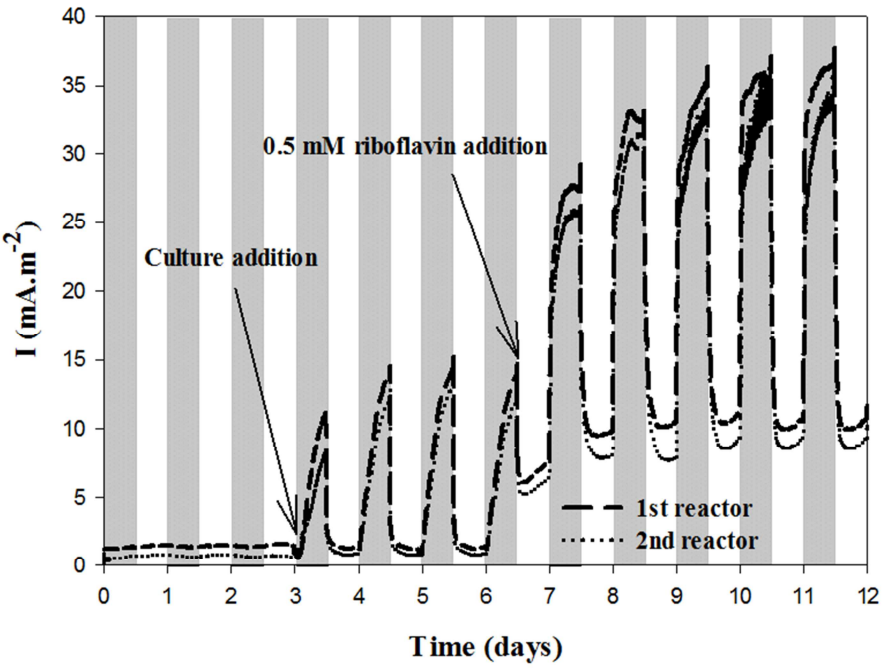

Figure 3.S2 Effect of cultures and riboflavin additions to anodic current profile in two identical bottle photoreactors. The dark and white bars symbolize dark and light phase, respectively

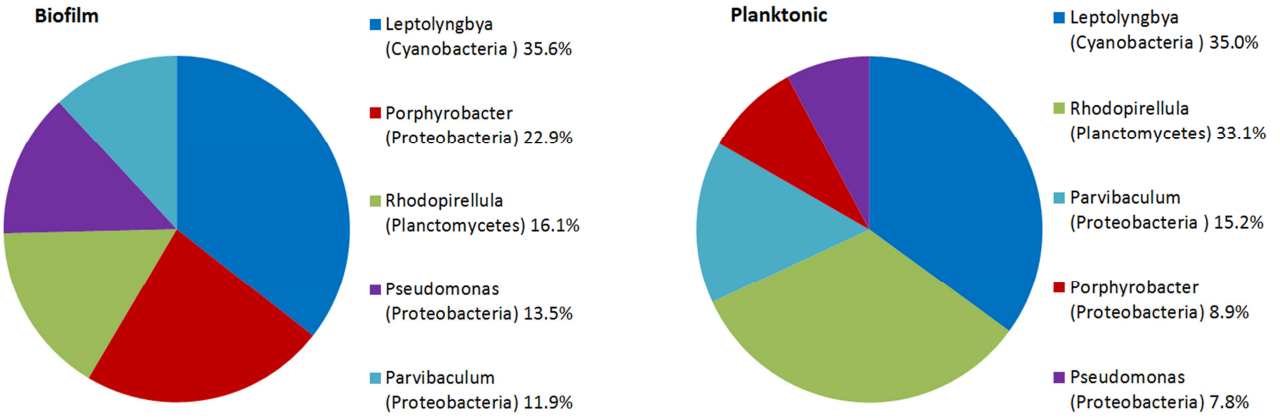

Figure 3.S3 16S-rRNA gene pyrosequencing analysis of bacteria taken from one of the two identical flat-plate photoreactors after 12-month exposure to RF and +0.6 V vs. SHE poised anode

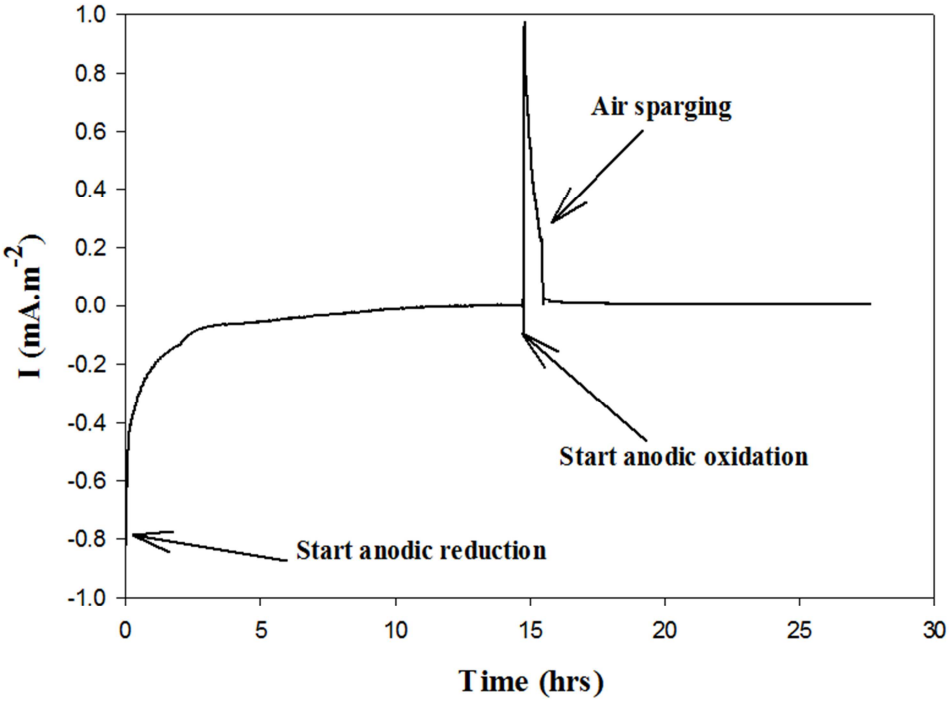

Figure 3.S4 Current profiles of redox reactions of 0.5 mM riboflavin in absence of both culture and illumination. Cathodic reduction and anodic oxidation were driven at poised-anode value of -0.22 V and + 0.6 V vs SHE, respectively in the absence of DO (continuous sparging of N_2). Experiment duplication resulted in consistent profile

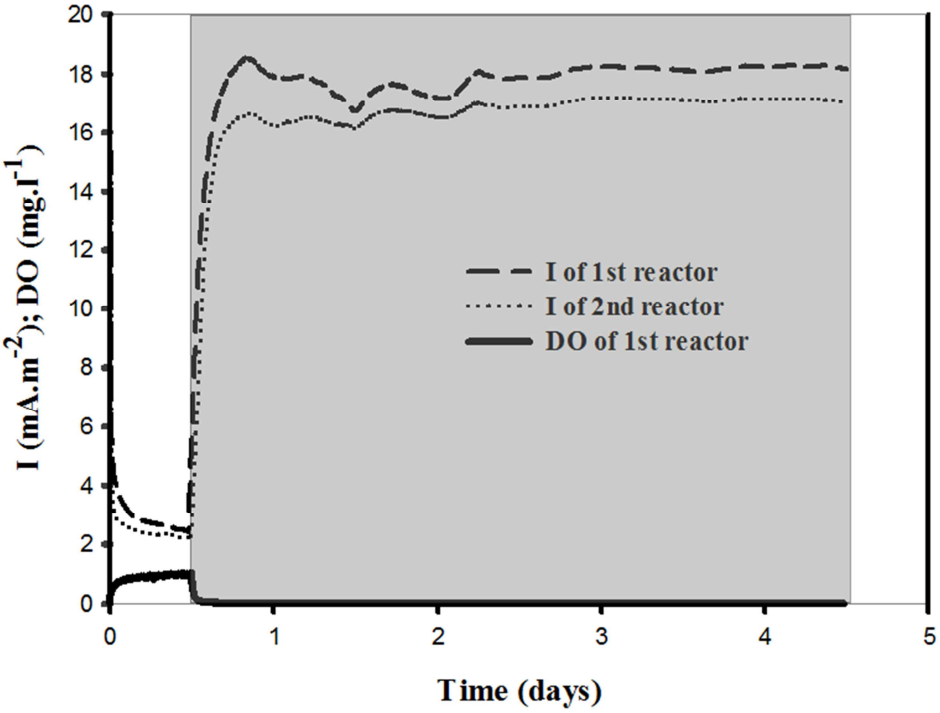

Figure 3.S5 Anodic current profile during four days dark phase of two identical flat-plate photoreactors (I of 1st and 2nd reactors, E = +0.6 V vs. SHE) and DO profile only measured in the 1st reactor, after >12 months exposure to riboflavin

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Chapter 4

Fully reversible current driven by a dual marine photosynthetic microbial community

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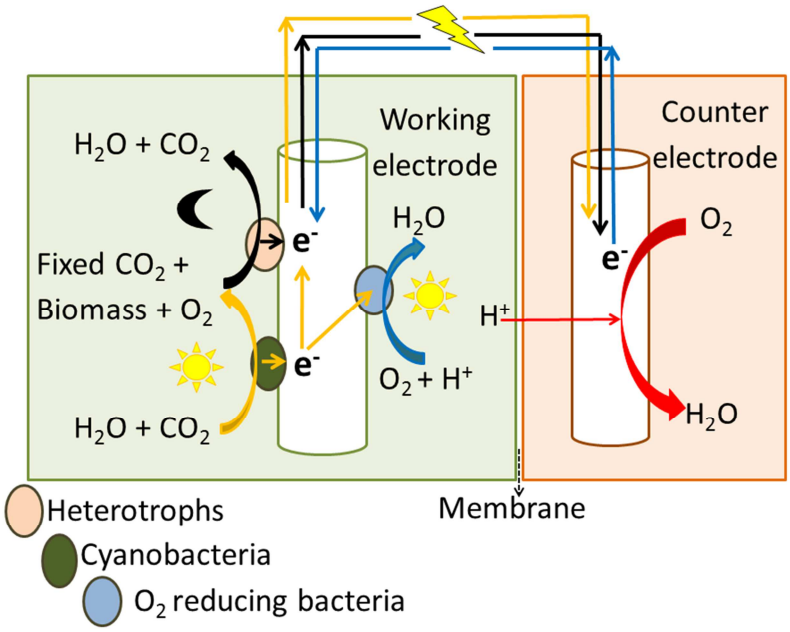
The electrochemical activities of two seawater microbial consortia were investigated in three-electrode bioelectrochemical cells. Two seawater inocula – from the Sunshine Coast (SC) and Gold Coast (GC) shores of Australia – were enriched at +0.6 V vs. SHE using 12/12 h day/night cycles. After re-inoculation, the SC consortium developed a fully-reversible cathodic/anodic current, with a max. of –62 mA m^{-2} during the day and +110 mA m^{-2} at night, while the GC exhibited negligible daytime output but +98 mA m^{-2} at night. Community analysis revealed that both enrichments were dominated by cyanobacteria, indicating their potential as biocatalysts for indirect light conversion to electricity. Moreover, the presence of γ-proteobacterium *Congregibacter* in SC biofilm was likely related to the cathodic reductive current, indicating its effectiveness at catalysing cathodic oxygen reduction at a surprisingly high potential. For the first time a correlation between a dual microbial community and fully reversible current is reported.

Graphical abstract



Highlights

- Fully reversible currents were observed only with a dual microbial community.
- Cathodic oxygen reduction occurs at high working electrode potential.
- Cathodic current is likely associated with γ-proteobacterium *Congregibacter*.
- Anodic current reflects biofilm capability to indirectly convert light to electricity.
- The highest solar bioanode (>100 mA m^{-2})/oxygen biocathode currents (>1000 mA m^{-2}).

Keywords: Reversible current; Cathodic current; Biophotovoltaics; Microbial solar cell; γ-proteobacterium *Congregibacter*

4.1 Introduction

Microbial solar cells (MSCs), also known as biophotovoltaics (McCormick et al. 2015), are bio-electrochemical systems (BESs) that convert solar energy into electrical energy through bio-electrocatalytic reactions driven by photosynthetic microorganisms. During oxygenic photosynthesis, solar energy is utilised to split water to oxygen, protons and electrons. These electrons are used by microorganisms for CO_2 fixation to form organic compounds (excreted and/or stored) and new cell material. Alternatively, they may be transferred extracellularly to a polarised anode (McCormick et al. 2011; Pisciotta et al. 2011). In MSC-type devices, the electrons then flow from anode to cathode where they reduce an electron acceptor, typically oxygen (Mateo et al. 2014).

Two-chamber mediatorless MSCs have been previously shown to exploit photosynthesis to generate anodic current catalysed by single species (Madiraju et al. 2012; Raman and Lan 2012) or photosynthetic microbial consortia (Cao et al. 2008) without added organic carbon sources. Other studies found instead that some photosynthetic microbial consortia in mediatorless MSCs were able to generate cathodic current during the day and anodic current during the night i.e. reversible current (He et al. 2009; Strik et al. 2010). This puzzling phenomenon may be used to overcome the problematic formation of a pH gradient across the membrane between anode and cathode (Blanchet et al. 2014; Strik et al. 2010). Moreover, polarity reversal of MFC-electrodes enhanced the anodic and cathodic power densities by 58% and 36%, respectively, when compared to non-reversing systems and led to a reduction in the use of a chemical buffering (Li et al. 2014).

This study aims to correlate the generation of reversible current with microbial community composition. For this purpose, two seawater biofilms enriched from different inocula were utilised in working electrode chambers for approximately 2 months at a poised electrode potential of +0.6 V vs. standard hydrogen electrode (SHE). We show that cathodic current during the day and anodic current during the night were biologically generated at the same electrode without input of exogenous organic compounds or redox mediators. Two distinct microbial consortia performed differently in terms of electrochemical activities, which appear to be related to the presence of distinct functionalities in the related biofilms. Furthermore, we show that with a dual microbial community comprising cyanobacteria and strong cathodic oxygen reducers (such as γ-proteobacteria), fully reversible currents are observed at a high working electrode potential of +0.6 V vs. SHE. The results provide more fundamental information to understand the phenomenon of reversible currents, which will be useful towards future designs of microbial solar cells.

4.2 Methods

4.2.1 Electrochemical cells setup

The experiments hereby presented were carried out using two dual-chamber bottle-type reactors. The reactors were constructed using 250 cm^3 borosilicate bottles as main chambers (200 cm^3 effective volume) each hosting a working electrode and a pipette tip as counter electrode chamber (6 cm^3 effective volume), immersed in the working electrode solution (Figure 4.S1, Supplementary material), and separated from the latter by a 4.9 cm^2 cation exchange membrane (CEM) (Ultrex CMI-7000, Membrane International). A 24 cm^2

rectangular piece of graphite felt was used as the working electrode (dimensions: 60 × 40 × 1 mm, Morgan Industrial Carbons, Australia) and pressed against a Titanium mesh (mesh size 1 mm × 2 mm, thickness 0.15 mm – Kaian Metal Wire Mesh Co. Ltd, China) that covered the whole working electrode surface for current collection. Both membrane and graphite felt were used as provided by the suppliers. Furthermore, each working electrode chamber also had an Ag/AgCl reference electrode (sat. KCl, +0.197 V vs. SHE), a pH probe (miniCHEM-pH, Australia) and a dissolved oxygen (DO) probe (O2-4100-e, Mettler Toledo, Germany) immersed in the medium. Titanium wires (length 4 cm, diameter 0.5 mm, Advent Research Materials, UK) were employed as counter electrodes. The reactors were placed in a custom made dark box with two built-in ventilation fans to maintain room temperature (24 ± 1 ^{o}C) and two white fluorescent lamps to illuminate the working electrode side of the reactors at 29 Wm^{-2}.

4.2.2 Photosynthetic culture enrichment

Two seawater inocula at pH 7.7–8.2 were taken from the Sunshine Coast (SC) and Gold Coast (GC) shores (eastern coast of Queensland, Australia), collected in depth of 5–30 cm below water surfaces using 15-L sterilised plastic container and stored at 4 ^{o}C before use. Both inocula were separately added to the working electrode chambers of two previously-sterilised electrochemical cells (described above) by mixing 50% of each sea water sample with filtered modified F2 medium (Pisciotta et al. 2010). To enable capturing all possible electron transfer pathways, the working electrode was poised at +0.6 V vs. SHE. Approximately 2 months after inoculation, the developed photosynthetic microbial biofilms were individually scraped from the whole surface of SC and GC working electrodes and used as sole sources for the re-inoculation of two new reactors (hereafter referred to as ‘‘re-inoculated reactors’’), which were operated at the same conditions for 25 days. Throughout operation, the filtered modified F2 medium was continuously supplied to the working electrode side of both reactors using a syringe pump at a flow rate of 1.04 mL h^{-1}, equivalent to a hydraulic retention time (HRT) of 8 days. This medium contained 37.6 g Sea salt (Ocean Nature, Aquasonic Pty Ltd, Australia); 75 mg $NaNO_3$; 5 mg $NaH_2PO_4 \cdot H_2O$; 0.046 mg $ZnSO_4 \cdot 7H_2O$; 0.304 mg $MnSO_4 \cdot H_2O$; 0.015 mg $Na_2MoO_4 \cdot 2H_2O$; 0.028 mg $CoSO_4 \cdot 7H_2O$; 0.014 mg $CuCl_2 \cdot 2H_2O$; 9.2 mg $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$; 8.8 mg $Na_2EDTA \cdot 2H_2O$ and 3.36 g $NaHCO_3$ as a buffering agent per litre at pH 7.5 ± 0.2. The counter electrode solution consisted of phosphate buffer (6 g Na_2HPO_4; 3 g KH_2PO_4) and 6 g NaCl per litre at pH 8.0 and was replaced on a daily basis to prevent excessively-high pH. A magnetic stirrer was used to continuously mix the working electrode medium at 500

rpm (magnetic bar diameter 0.5 cm and length 2 cm). Illumination (measured using an IR day sensor PS-2148, PASPort™) was maintained at 12 h/12 h day/night pattern and each working electrode potential was poised at +0.6 V vs. SHE with a multichannel potentiostat (CHI-1000B, CH Instruments). Unless otherwise specified, a humidified 97% N_2/3% CO_2 gas mix (BOC, Australia) was used at a rate 0.2 L min^{-1} to strip out photosynthetic DO and to provide a carbon source for photoautotrophic microorganisms in the working chamber solution.

4.2.3 Electrochemical analysis

The electrochemical performance of the working electrodes was tested by two methods, potential-step chronoamperometry and cyclic voltammetry (CV) using the multichannel potentiostat. Multi-step chronoamperometry was performed at a potential range of –0.4 to +0.6 V vs. SHE; each step was held for 2 h. Cyclic voltammetry was run at a scan rate of 1 mV s^{-1} (Harnisch and Freguia 2012) with a scan range of –0.4 to +0.6 V vs. SHE and the voltammogram was compared to that recorded on an abiotic control reactor running separately in identical configuration and operational conditions. The current densities are reported normalised to the projected surface area of the working electrode (24 cm^2).

4.2.4 DNA extraction and amplicon sequencing

Inoculum samples were collected by centrifuging 14 L sea water samples at the time of inoculation. Biofilm samples were collected from the re-inoculated reactors by scraping the whole working electrode surfaces on the day phase (day 25) after CV and step-chronoamperometry were conducted. Genomic DNA was extracted from 25 mg of either the collected inoculum and biofilm samples by FastSpin for Soil Kit (MP-Biomedicals, USA) according to the manufacturer's protocol. 300 ng DNA of each sample were provided to the Australian Centre for Ecogenomics (ACE) at the University of Queensland for 16S amplicon pyrosequencing by Illumina Miseq Platform using 926F and 1392wR primer sets (Engelbrektson et al. 2010).

4.2.5 Amplicon sequencing data analysis

After sequencing, raw paired reads were first trimmed by Trimmomatic (Bolger et al. 2014) to remove short (less than 190 bp) and low quality reads (lower than Phred-33 of 20). The trimmed reads were then assembled by Pandaseq (Masella et al. 2012) with default parameters and the adapter sequences were removed by FASTQ Clipper of FASTX-Toolkit (Pearson et al. 1997). The joined high quality sequences were then analysed by

QIIME v1.8.0 (Caporaso et al. 2010) using open-reference OTU picking strategy and assigned taxonomy by uclust (Edgar 2010), against greengenes database (13_05 release). Normaliser (https://github.com/minillinim/Normaliser) was used to find a centroid normalised OTU table. Major OTUs (with >1% relative abundance in each sample) were used to generate heatmaps with the 'pheatmap' package with R, version 3.0.1 (R Development Core Team, 2013) (http://www.R-project.org).

4.2.6 Microscopic analysis

Collected samples (see Section 2.4 DNA extraction and amplicon sequencing) were fixed with 4% paraformaldehyde (PFA) for 2 h on the day of collection, then washed with phosphate buffered saline (PBS) and resuspended in 1:1 100% ethanol: PBS solution. Fluorescent in situ hybridization (FISH) was carried out on fixed samples (Amann et al. 2001). General bacteria probe (EUB mix-FITC) and general eukarya probe (EUK mix-Cy3) (Table 4.S1, Supplementary material (SM)) were applied. The slides were then viewed under the Axioscope LSM510 confocal microscope (Zeiss, Germany). Biovolume fraction of bacteria to total biofilm community was quantified by Daime software as described in our previous study (Darus et al. 2014). Gram stain was furthermore performed according to Beveridge (2001).

4.3 Results and discussion

4.3.1 Photosynthetic biofilms performing reversible current

Following the inoculation of GC and SC samples (day 8 and 10, respectively in Figure 4.1A), the current profiles started to fluctuate following a day/night pattern after 12 and 14 days, respectively (day 22, Figure 4.1A). With further development of electrogenic activity in both reactors, observable green microbial biofilms were established on the light-facing sides of the working electrodes. The current was anodic during the night and negligible or even slightly cathodic during the day. The cathodic current was first observed in the SC reactor three days after the first current fluctuation (day 25, Figure 4.1A), but no cathodic current was observed for the GC reactor even after 46 days.

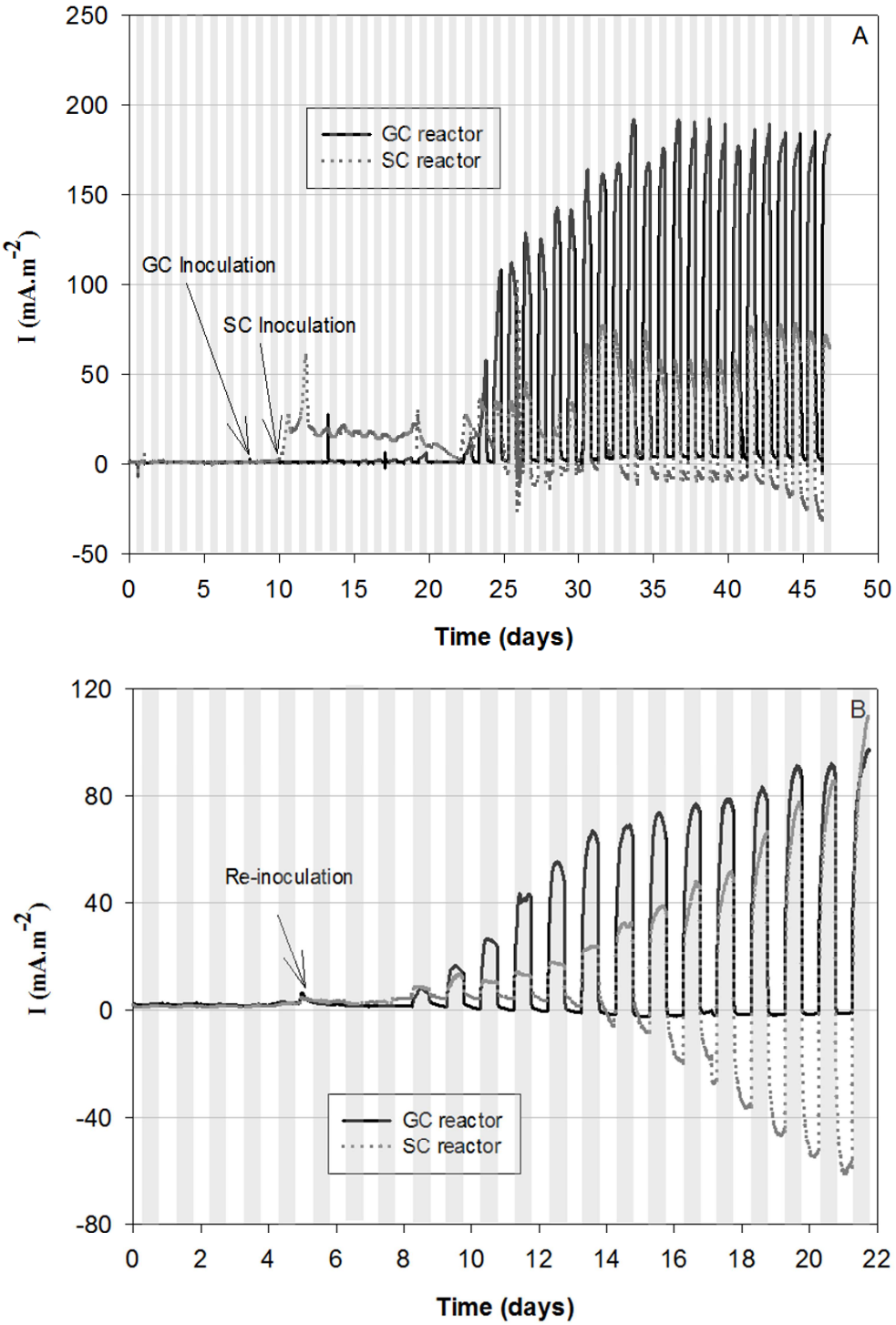


Figure 4.1 Chronoamperometric current profiles of two different seawater MSCs at a poised working electrode potential of +0.6 V vs. SHE. (A) Reactors inoculated with the Sunshine Coast (SC) and Gold Coast (GC) samples. (B) Reactors re-inoculated with biofilm samples taken from the previously-enriched biofilms shown in A. The dark and clear areas indicate night and day phases, respectively

Subsequently, when samples from these green biofilms of SC and GC reactors were used to re-inoculate two new reactors, a faster development was observed for both biofilms and

day/night current fluctuations were observed in both cases after just 3 days (Figure 4.1B). The faster performances must be credited to the fact that both biofilm samples used for the re-inoculation were already electrochemically active. Following the re-inoculation, the SC reactor started to generate cathodic currents 6 days after the first current fluctuation (day 14, Figure 4.1B), while daytime cathodic currents were minimal for the GC reactor. At an applied potential of +0.6 V vs. SHE, SC and GC working electrodes produced peak cathodic currents of 62 mA m^{-2} and 4 mA m^{-2} when illuminated and anodic currents of 110 mA m^{-2} and 98 mA m^{-2} in the night, respectively. This reversible current is significantly higher than previously achieved in mediatorless mixed photosynthetic cultures for anodic and cathodic current, respectively: 6.9 mA m^{-2} and 5.8 mA m^{-2} (He et al. 2009), 10.0 mA m^{-2} and 3.3 mA m^{-2} (Strik et al. 2010). As there was no external addition of organic compounds to the medium, the night time effect may be attributed to the oxidation of photosynthetically produced organic compounds, possibly excreted and/or stored, as well as cell debris (Fu et al. 2009), while the day time effect may be due to the previously reported effect of evolved oxygen (Darus et al. 2014; Strik et al. 2010).

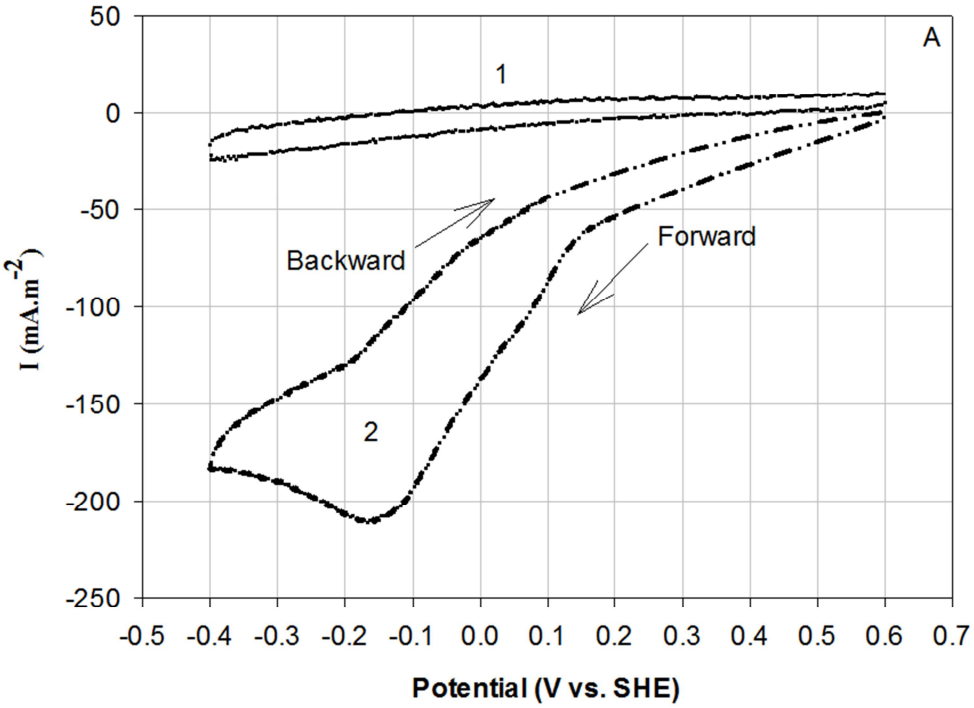
The facts that anodic and cathodic current fluctuations were not observed before inoculation, when the working electrode chambers contained only continuously aerated medium (days 0–8, Figure 4.1A and days 0–5, Figure 4.1B), confirms that biological activities were responsible for the reversible current phenomenon. A faster start-up period after re-inoculation with the pre-enriched samples corroborates this conclusion.

Furthermore, there was no observable presence of green suspended microorganisms in both either anolyte or catholyte during the whole experimental period, both visually remaining clear over time. When the counter electrode chambers (including respective buffers) were replaced with fresh sterile ones, no changes in current profiles ensued, excluding a role of the counter electrodes in the current profiles. In this study, the biofilms growing on the graphite felt electrodes were therefore fully responsible for the observed reversible currents.

4.3.2 Biocatalytic oxygen reduction at high potential by enriched SC biofilms

To further study the cathodic current generation by SC biofilm, cyclic voltammetry was conducted on the SC re-inoculated reactor at the end of day phase (day 22, Figure 4.1B). During the day, the cyclic voltammogram of the SC working electrode (in this case effectively a cathode) displayed a cathodic current onset at a potential of approximately

+0.6 V vs. SHE (Figure 4.2A), as a result of oxygen reduction: DO concentrations changed from below detection limit in the night, to 0.5–0.7 mg L^{-1} in the day, measured at the end of day/night pattern at maximum current generation. The cathodic process appears kinetically limited down to a potential of +0.1 V vs. SHE, when a second mechanism likely kicks into boost oxygen reduction rates (Figure 4.2A). Measurement of DO during the cyclic voltammetry revealed that DO was indeed being depleted during the experiment (Figure 4.S2).



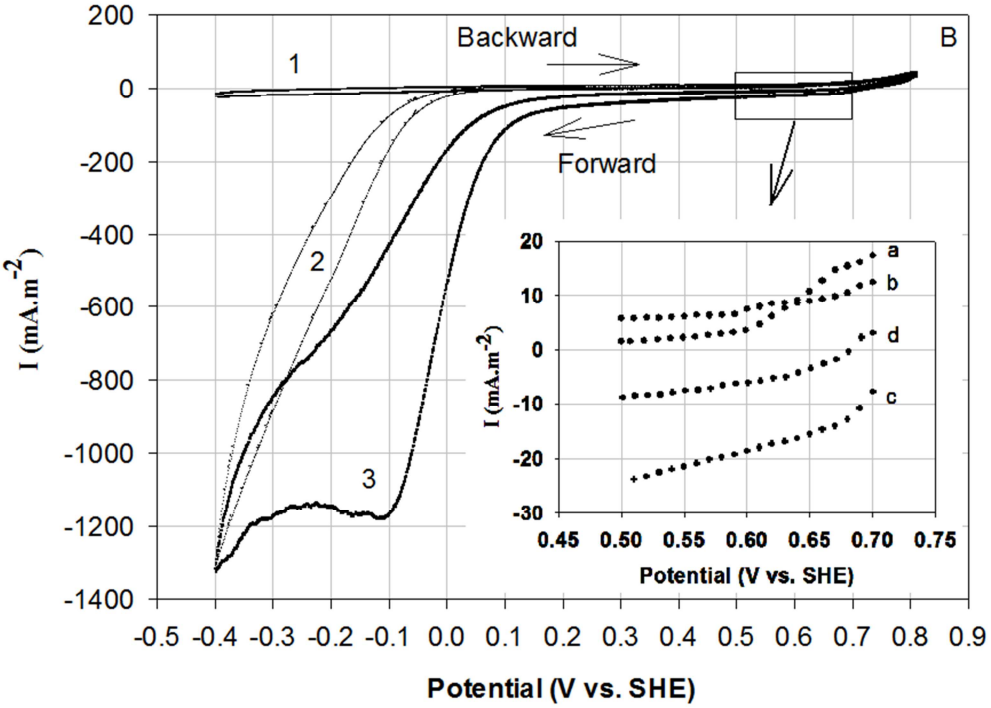


Figure 4.2 Cyclic voltammograms in the day at scan rate 1 mV s^{-1}, (A) at potential range –0.4 to +0.6 V vs. SHE for control (1) and the SC re-inoculated reactor (2); (B) at potential range –0.4 to +0.8 V vs. SHE for control (1), aerated control (2) and aerated-SC re-inoculated reactor (3); inset B: forward (a) and backward (b) scans of aerated control, forward (c) and backward (d) scans of aerated-SC re-inoculated reactor, at potential range +0.5 to +0.7 V vs. SHE

To determine the full potential of this biofilm as oxygen biocathode catalyst, the day phase CV was repeated with active aeration at a rate of 0.2 L min^{-1} in the working electrode solution. With active aeration, the onset was unchanged but far larger current densities were achieved due to the absence of mass transfer limitations, peaking at 1200 mA m^{-2} at –0.1 V vs. SHE (Figure 4.2B). The abiotic control exhibited far lower onset potential (below 0 V vs. SHE) (Figure 4.2B), as previously shown with aerated-uncatalysed graphite felt (Freguia et al. 2010) and glassy carbon (Liu et al. 2013).

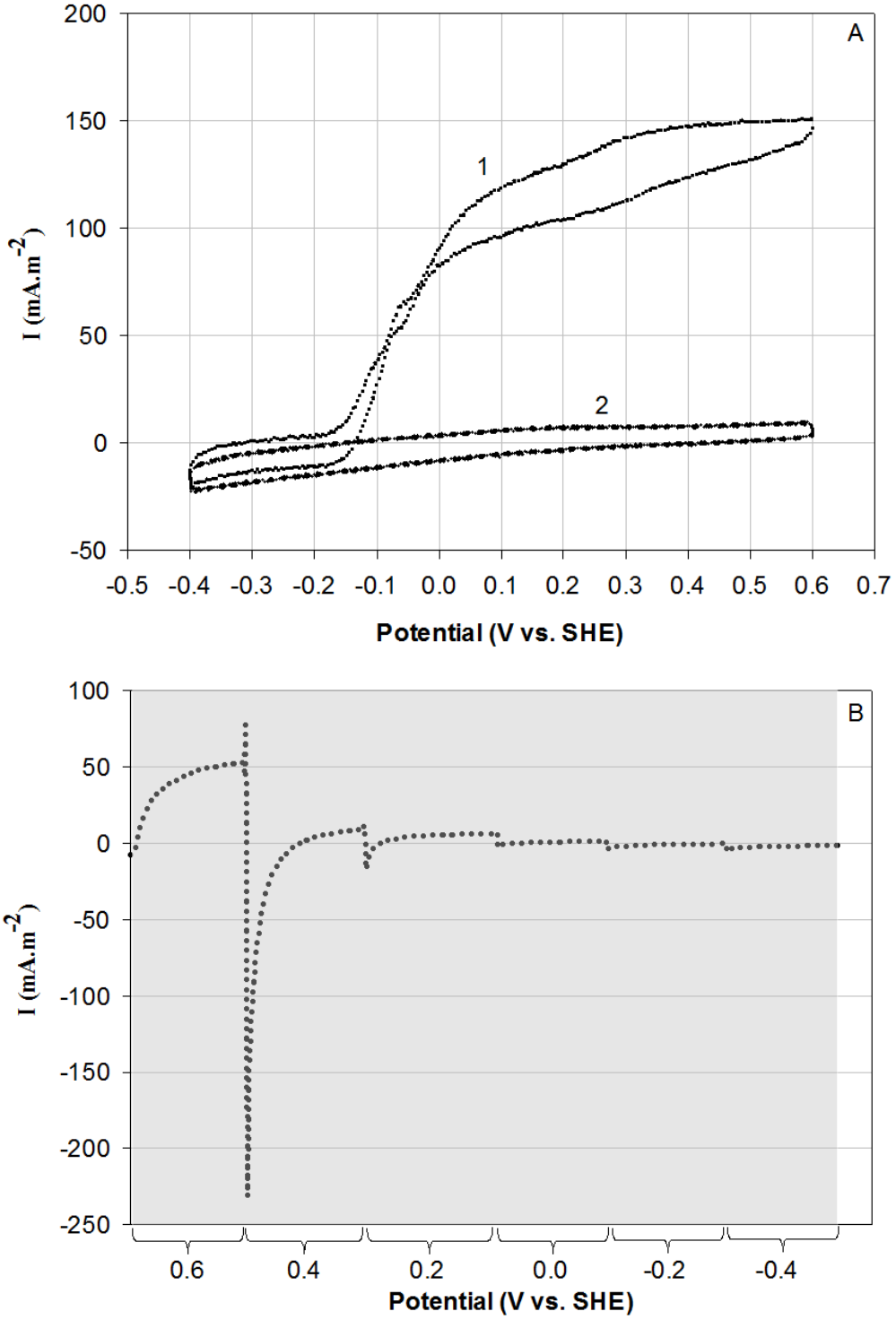


Figure 4.3 (A) Dark phase-cyclic voltammograms of the SC re-inoculated reactor (1) and control (2) at scan rate 1 mV s^{-1}, at potential range –0.4 to +0.6 V vs. SHE; (B) potential step-chronoamperometry of GC re-inoculated reactors at poised anodic potentials of +0.6, +0.4, 0.2, –0.0, –0.2 and –0.4 V vs. SHE each held for 2 h in night phase

4.3.3 Anodic biocatalysis by enriched SC and GC biofilms

Voltammograms of the SC working electrode recorded at the end of night phase (day 21, Figure 4.1B) (in this case effectively an anode) showed that the SC biofilm started to

produce anodic current at potential between –0.2 and –0.1 V vs. SHE, while such a current was not produced in the anoxic sterile control Figure 4.3A). For the GC reactor, a much higher anodic onset (around +0.2 V vs. SHE) (Figure 4.3B) was observed from potential step-chronoamperometry conducted during night time at day 24 (not shown in Figure 4.1B). This difference highlights the dissimilar bio-electrocatalytic activities of GC and SC bioelectrodes, which may be attributed to differences in the microbial biofilm communities, as shown in the following section.

4.3.4 Microbial communities of inocula and enriched biofilms

To identify the biofilm communities, total 126,565 sequences were recovered from SC, GC inocula and biofilms. Pyrosequencing illustrated the presence of bacteria and unassigned OTUs (considered as eukaryotes) in all samples. Reduced Shannon diversity was observed in both biofilms (Figure 4.S3) indicating the bacterial community shifted from inocula with high diversity towards mainly cyanobacteria in the biofilms. Three main morphologies observed in FISH images include filamentous (in both biofilms, Figure 4.S4A–F), coccal and large irregular cells (in GC biofilm, Figure 4.S4D–F). Figure 4.S4A and S4D represent EUB mix-probed bacteria in green (FITC filter). Both cyanobacteria and algae contain Chlorophyll which autofluorescence in Cy3 filter (Figure 4.S5B) (Anagnostidis 1989; Komárek 2007). Hence, Figure 4.S4B and S4E represent autofluorescent bacteria, autofluorescent eukaryote and EUK mix-probed eukaryote in red (Cy3 filter). Finally Figure 4.S4C and S4F represent the whole communities. Cyanobacteria are presented in yellow (Figure 4.S4C and S4F) as an overlap of EUB mix probe binding in FITC filter (Figure 4.S4A and S4D) and autofluorescence in Cy3 filter Figure 4.S4B and S4E) in both biofilms. Cyanobacteria in SC biofilm, which refer to filaments (Figure 4.S4C), were clearly shown to dominate the biofilm community. Although filaments and cocci showed weak autofluorescence in FITC filter (Figure 4.S5A), they were confirmed to be gram negative bacteria by gram stain (data not shown). Figure 4.S4F in turn shows that algae, which refer to large irregular cells, were observed in GC biofilm in red (in Cy3 filter) as autofluorescence and EUK mix probe binding. The algae were present in minor quantities in GC biofilm as quantified by Daime software while cyanobacteria, which refer to filaments and cocci accounted for 89.4% of the total biovolume fraction (100.0% and 9.7% for congruency and standard deviation).

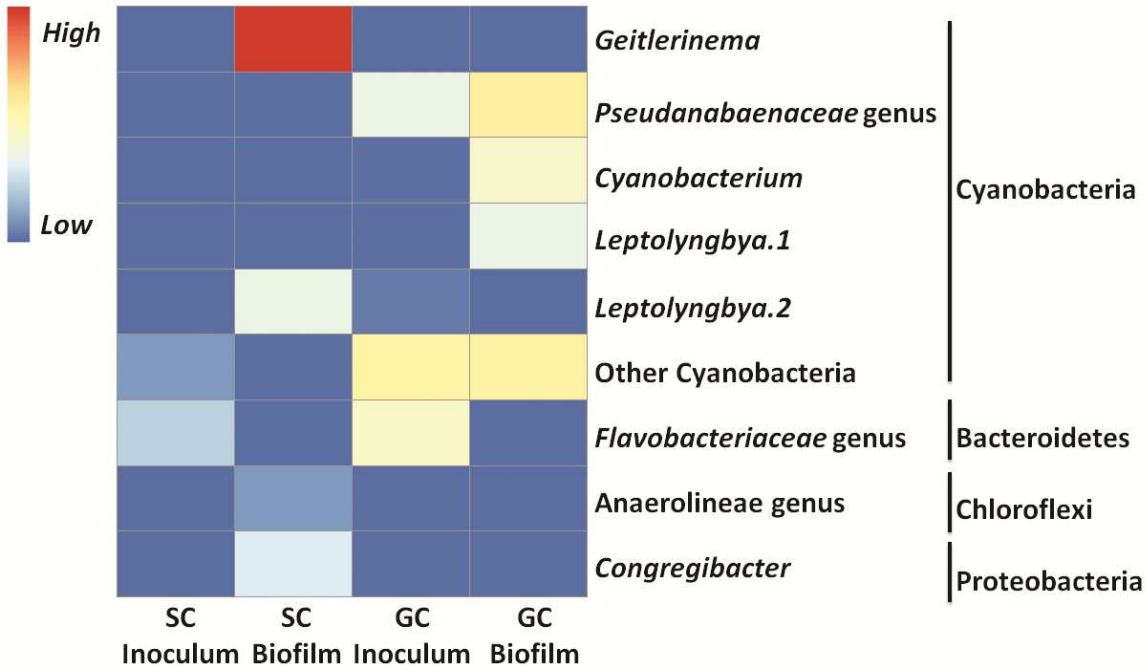


Figure 4.4 Heat map of major abundant OTUs in each sample. OTUs with >1% relative abundance in each sample were selected and labelled as either genus identification or the lowest taxonomy classification (e.g., Anaerolineae genus refers to a genus in class Anaerolineae). OTUs with <1% abundance in phylum Cyanobacteria were summarised and presented as other Cyanobacteria. The colour intensity in each cell shows the relative abundance (after square root transformation) of each OTU in a sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SC biofilm was enriched in *Geitlerinema* (88%) and *Leptolyngbya 2* (10%) within the phylum Cyanobacteria from undetectable amount of cyanobacteria in the inoculum. Other Cyanobacteria genus (individual relative abundance <1%) were present in low abundance (<10%). Regarding other bacteria, there are fair amounts of OTUs affiliated to γ-proteobacterium *Congregibacter* (8%) and Chloroflexi Anaerolineae (1%) detected (Fig. 4). *Geitlerinema* and *Leptolyngbya* are filamentous (Anagnostidis 1989; Komárek 2007) and possibly represent the filaments observed in Figure 4.S4A–C.

The microbial community in GC inoculum is less diverse than SC inoculum with regards to cyanobacteria. The dominance of *Pseudanabaenaceae* genus remained in GC biofilm. *Cyanobacterium* and *Leptolyngbya 1* emerged in biofilm and replaced the *Flavobacteriaceae* genus. Large amount of other Cyanobacteria genus (individual relative abundance <1%) were observed in both inoculum and biofilm indicating a diverse

microbial community within the phylum Cyanobacteria. There were no other bacteria detected at >1% level.

Cyanobacteria are photoautotrophs known for their putative capability of anodic and cathodic extracellular electron transfer. The anodic current generation was previously shown via several mechanisms such as water splitting (Madiraju et al. 2012; Pisciotta et al. 2010; Raman and Lan 2012) and oxidation of endogenously stored photosynthetic substrate (Tanaka et al. 1988) or exogenous added substrate (Yagishita et al. 1999). Among the major cyanobacteria detected in our systems, only *Leptolyngbya* (Pisciotta et al. 2010) and *Pseudanabaenaceae* genus (Luimstra et al. 2014) were previously reported to be capable of performing anodic current. Considering the dominance of cyanobacteria in our systems including *Geitlerinema*, *Cyanobacterium* and other Cyanobacteria genus (<1% abundance), it is reasonable to speculate that cyanobacteria had an important role towards the observed anodic electrochemical activities.

Microcystis aeruginosa IPP within phylum Cyanobacteria was previously found to be capable of indirectly inducing cathodic processes under illumination, albeit at potentials below 0 V vs. SHE (Cai et al. 2013). High potential (> +0.3 V vs. SHE) cathodic oxygen reduction has thus far only been associated to the presence of γ-proteobacteria (Rothballer et al. 2015). In our work, γ-proteobacterium *Congregibacter* in SC biofilm may be the cause of the high potential cathodic catalytic activity of this bioelectrode. *Congregibacter* is an aerobic anoxygenic photosynthetic bacterium, able to internally transfer electrons via both photosynthetic and respiratory electron transport chains (Fuchs et al. 2007). *Congregibacter litoralis* was previously shown to be capable of catalysing oxygen reduction to water or hydrogen peroxide (Spring et al. 2009). Therefore, we can speculate that there is a strong correlation between the presence of γ-proteobacterium *Congregibacter* in the SC biofilm and its biocatalytic cathodic activity. In the GC biofilm, in which *Congregibacter* was not detected, only minimal biocathodic activity was observed.

Chloroflexi Anaerolineae are obligate anaerobes (Yamada et al. 2006) and their presence in SC biofilm may be explained by the possibly formed anaerobic layer of biofilm with a possible contribution to anodic current generation via oxidation of excreted photosynthetic substrate and cell debris.

The intensity of light exposure is a critical selective pressure for microbial community. A non-photosynthetic bacterium, *Flavobacteriaceae* genus that was present in high amount in both inocula, was replaced by cyanobacteria (Figure 4.4). In addition, the choice of inoculum may be another important factor that affects the microbial community and reactor performance. The high amount of cyanobacteria in GC inoculum may generate a competitive pressure that limits the growth of Geitlerinema and other bacteria such as *Congregibacter*, thus leading to a lack of development of cathodic biocatalysis by these bacteria.

So far, no reversible current generation has been reported for individual photosynthetic microorganisms, while such capability was previously observed in two-chamber MSCs in the presence of microbial consortia. This suggests the importance of functionally different communities for reversible electrochemical activity. Remarkably, SC bioelectrodes generated fully-reversible current at +0.6 V vs. SHE in the presence of a dual photosynthetic microbial community, comprising cyanobacteria and a putative cathodic oxygen reducer, γ-proteobacterium *Congregibacter*. The original inoculum appears to be paramount towards the development of these reversible solar bioelectrodes. Indirect photo-induced extracellular electron transfer by cyanobacteria during the night, most likely by oxidation of photosynthetic organic compounds (Madiraju et al. 2012; Pisciotta et al. 2010), appears to be a distinct feature of seawater cultures, indicating that this metabolic ability may be common in seawater environments. Knowledge of extracellular electron transfer pathways is required to further the understanding of reversible electrochemical performance for application in MSCs.

4.4 Conclusions

Fully reversible current generation at microbial solar electrodes is microbe-dependent and requires a dual community within the biofilm, comprising cyanobacteria for anodic current and high potential oxygen reducers for cathodic current generation. It is noteworthy that the onset of biocatalytic oxygen reduction occurs here at high potential, making this biocatalyst comparable (in terms of onset) with some purified enzymes for cathodic oxygen reduction. The reversible electrode developed in this work generated some of the highest current densities reported to date for both solar bioanodes (>100 mA m^{-2}) and for oxygen reducing biocathodes (>1000 mA m^{-2}).

4.5 Acknowledgements

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4.6 Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.06.055.

Table 4.S1 Sequences of FISH probes, EUB mix-FITC (Eub338, Eub338II, Eub338III) and EUK mix-Cy3 (Eukb1193, Eukb503, EUKb310) used in experiment

Target population(s)	FISH probe-fluorochrome	Probe sequences(5'-3')
Bacteria	Eub338	5'-GCTGCCTCCCGTAGGAGT-3'
	Eub338II	5'-GCAGCCACCCGTAGGTGT-3'
	Eub338III	5'-GCTGCCACCCGTAGG TGT-3'
Eukarya	Eukb1193	5'-GGGCATMACDGACCTGTT-3'
	Eukb503	5'-GGCACCAGACTKGYCCTC-3'
	EUKb310	5'-TCAGGCBCCYTCTCCG-3'

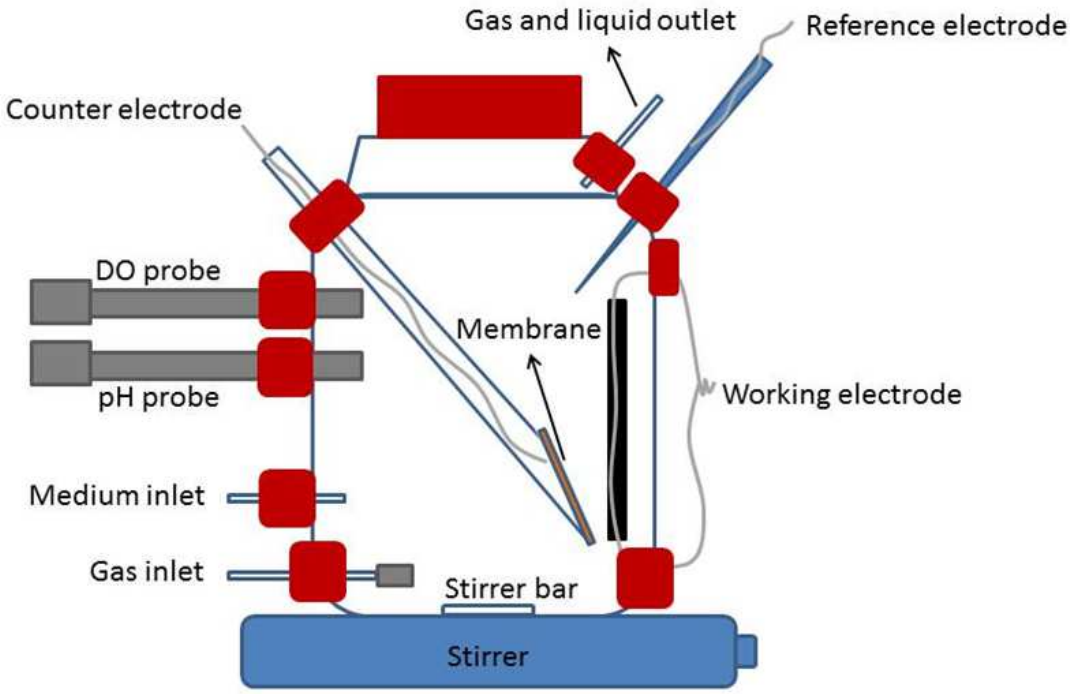

Figure 4.S1 Picture of two-chamber bottle reactor

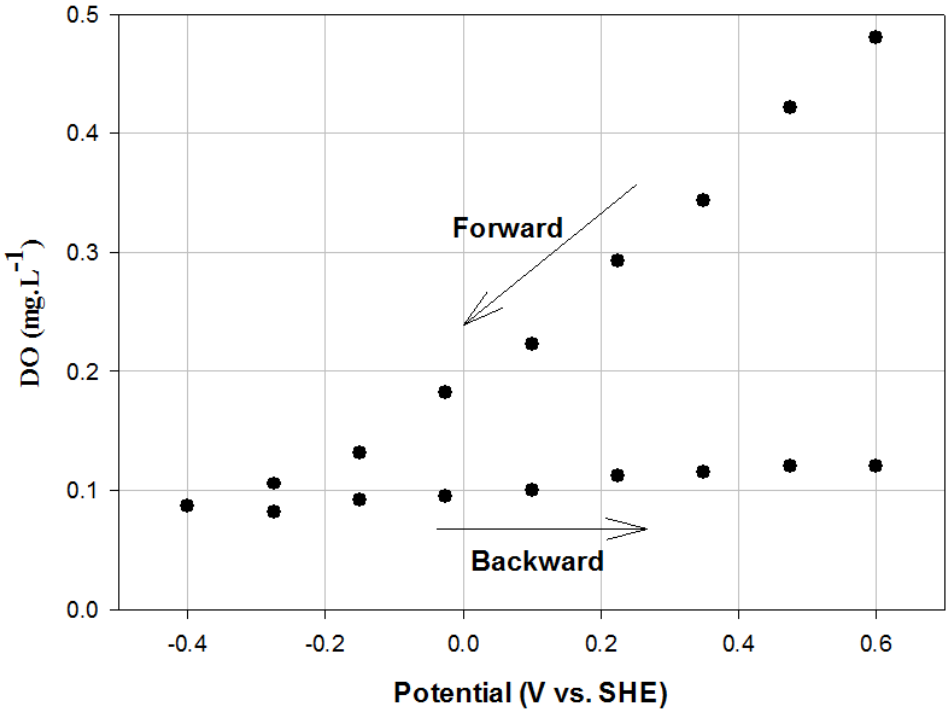

Figure 4.S2 Dissolved oxygen profile during cyclic voltammetry of the SC reinoculated reactors in day phase conducted at scan rate 1 $mV.s^{-1}$, at potential range -0.4 to +0.6 V vs SHE

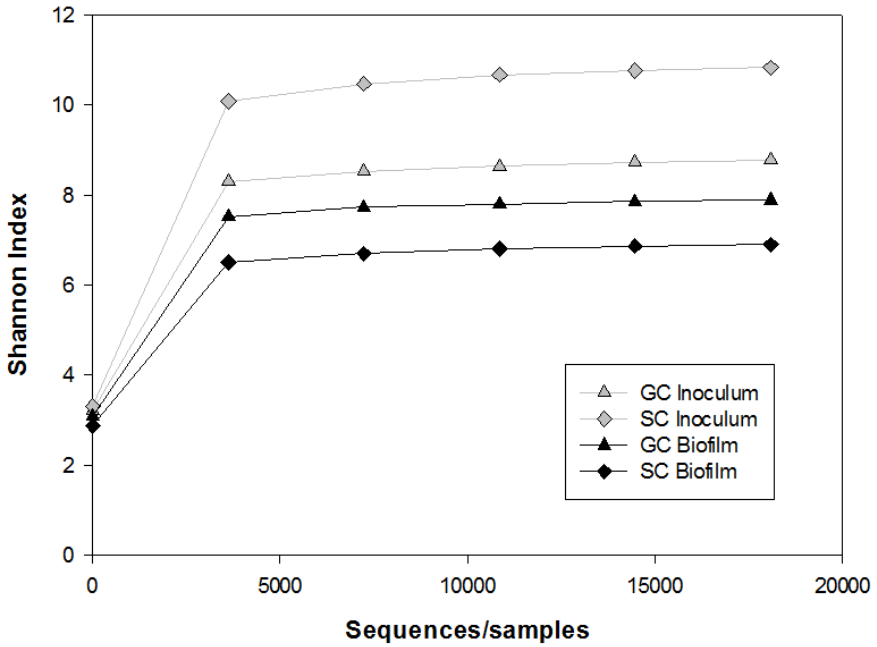

Figure 4.S3 Rarefaction curve comparing the Shannon index of the 16S rRNA gene sequences between inoculum (marked as grey) and biofilm (marked as black). Diamond and triangle lines indicate the sequencing from SC and GC respectively. The maximum number of sequences per samples is 20,000 as used in normalization step

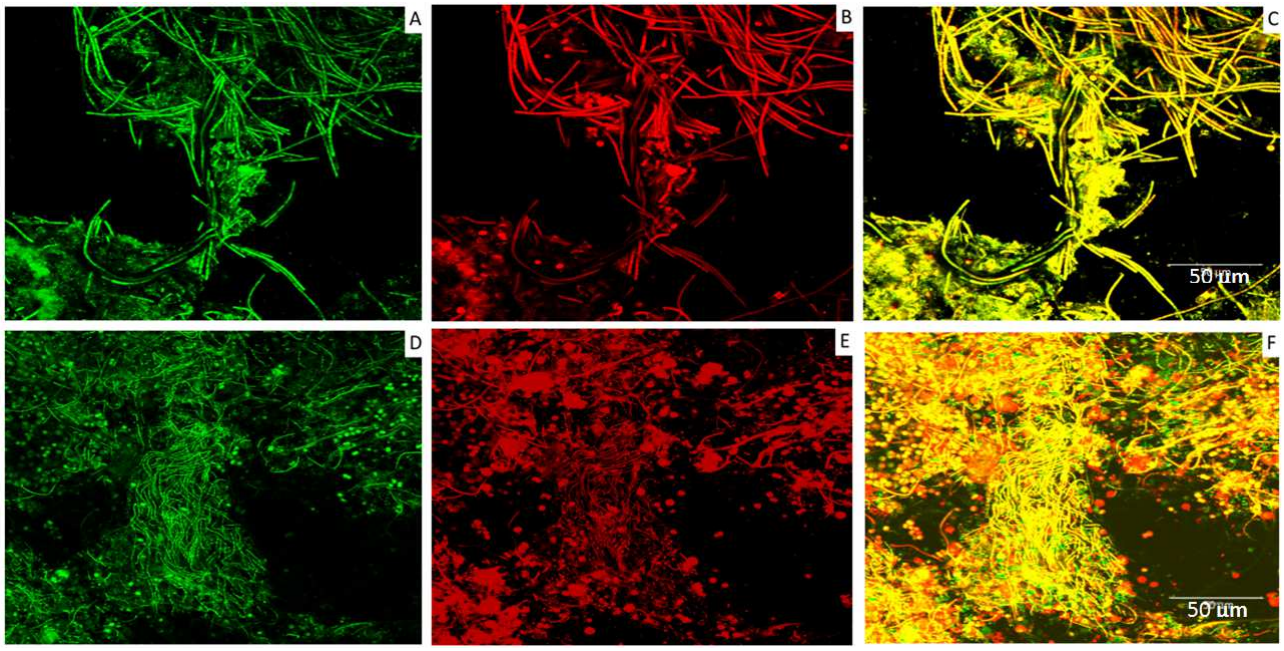

Figure 4.S4 FISH images of biofilm communities from the same microscopic field from a sample of the SC (A, B, C) and GC (D, E, F) biofilms. (A, D) EUB mix-probed bacteria in FITC; (B, E) autofluorescent and probed bacteria and eukaryote in Cy3; (C, F) whole community consisting of EUB mix-probed bacteria and EUK mix-probed eukaryote

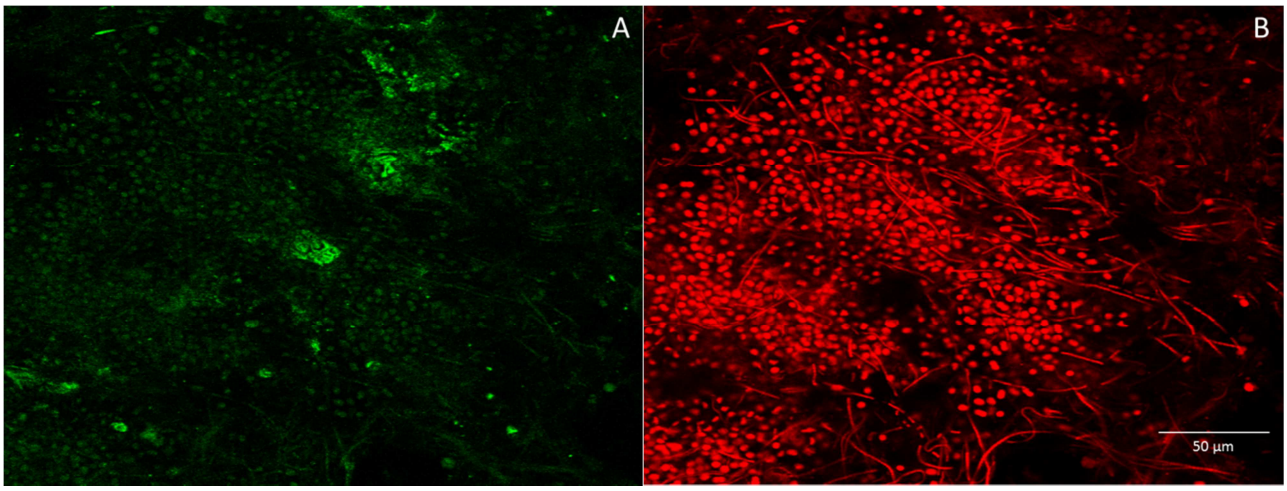

Figure 4.S5 Images of autofluorescent filaments and cocci in GC biofilm from the same microscopic field: in FITC (A) and Cy3 (B) filters

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Chapter 5

Marine phototrophic consortia transfer electrons to electrodes in response to reductive stress

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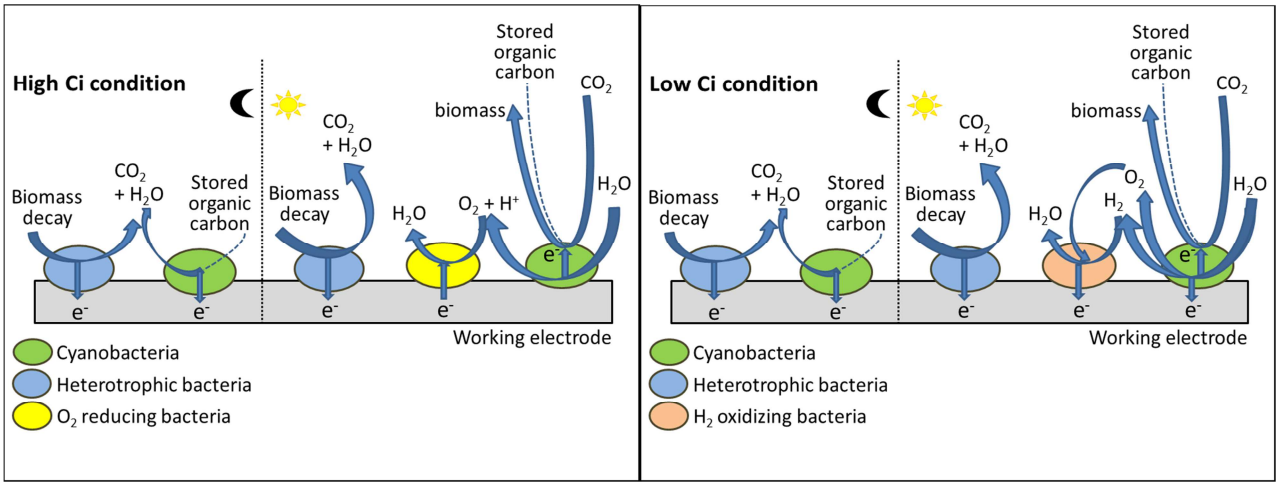
This work studies how extracellular electron transfer (EET) from cyanobacteria-dominated marine microbial biofilms to solid electrodes is affected by the availability of inorganic carbon (Ci). The EET was recorded chronoamperometrically in the form of electrical current by a potentiostat in two identical photo-electrochemical cells using carbon electrodes poised at a potential of +0.6 V vs. Standard Hydrogen Electrode under 12/12 h illumination/dark cycles. The Ci was supplied by addition of $NaHCO_3$ to the medium and/or by sparging CO_2 gas. At high Ci conditions, EET from the microbial biofilm to the electrodes was observed only during the dark phase, indicating the occurrence of a form of night-time respiration that can use insoluble electrodes as the terminal electron acceptor. At low or no Ci conditions however, EET also occurred during illumination suggesting that, in the absence of their natural electron acceptor, some cyanobacteria are able to utilise solid electrodes as an electron sink. This may be a natural survival mechanism for cyanobacteria to maintain redox balance in environments with limiting CO_2 and/or high light intensity.

Graphical abstract



5.1 Introduction

Photosynthetic microorganisms normally utilise inorganic carbon (Ci) in form of CO_2 as electron acceptor. The Ci uptake in photosynthetic microorganisms is conducted through the intracellular CO_2 concentrating mechanism (CCM) to maximize the CO_2 fixation rate by accumulating ambient levels of CO_2 present in the natural environment (≤15mM) into high level HCO_3^- within the microbial cell up to 15-50 mM (Badger and Schreiber 1993; Miller et al. 1990). Interestingly, under reductive stress conditions such as low or nil Ci and/or strong illumination, photosynthetic microorganisms resort to photoprotective mechanisms for their photosynthetic machinery – particularly photosystem 1 (PS1) and photosystem 2 (PS2) – including dissipation of excess energy as heat (Horton et al. 1996; Müller et al. 2001). Cyanobacteria are also able to dissipate excess energy through quenching of excited state phycobilisomes (using orange carotenoid proteins (El Bissati et al. 2000; Kirilovsky 2007; Rakhimberdieva et al. 2010)), oxygen photoreduction to water in PS1 (Badger et al. 1985) and PS2 (Shimakawa et al. 2015), and photoprotection against reactive oxygen species (ROS) by ROS-scavenging enzymes in PS2 (Aro et al. 1993; Zhang et al. 2009).

Some studies have proposed that electrons are able to migrate from the cyanobacterial photosynthetic electron transport chain to insoluble extracellular electron acceptors via PS1 in the cases of *Synechococcus sp*. (Yagishita et al. 1993) and *Synechocystis sp*. PCC 6803 (McCormick et al. 2011) or via PS2 in *Nostoc* and *Lyngbya* (Pisciotta et al. 2011). This process is termed extracellular electron transfer (EET) and can be measured in the form of anodic electrical current if an electrically conductive solid (electrode) is selected as

the terminal insoluble electron acceptor. Anodic electrical currents from pure cyanobacterial cultures have been previously observed in photo-electrochemical cells under illumination (Pisciotta et al. 2010; Zou et al. 2009). In the presence of photosynthetic microbial consortia, anodic currents have also been observed, but only under dark conditions, putatively via oxidation of stored organic matter or cell lysis products (Darus et al. 2015; He et al. 2009; Strik et al. 2010). Illumination of these cultures resulted conversely in cathodic (reductive) current, caused by the reduction of photosynthetically-generated extracellular oxygen (Darus et al. 2015).

This work aims to investigate some of EET pathways in photosynthetic microbial consortia, particularly with the aim of elucidating the key pathways and conditions that facilitate this process. Our findings indicate that the absence of inorganic carbon (leading to excess reducing power inside the cells) triggers the diversion of electrons onto extracellular electron acceptors, including soluble compounds such as dissolved oxygen and – most remarkably – electrodes. This is a previously-undescribed pathway for dissipation of excess reducing power that may help maintain redox balance in photosynthetic microorganisms under reductive stress.

5.2 Methods and analyses

5.2.1 Experimental setup and operation

Two autoclaved dual-chamber bottle-type photo-electrochemical cells, of the type previously presented (see Darus et al. 2014; 2015), were utilised for these experiments for the total duration of 105 days. Briefly, each cell contained a 24 cm^2 piece of plain graphite felt as working electrode, a KCl-saturated Ag/AgCl reference electrode and a Titanium wire (length 4 cm, diameter 0.5 mm) as counter electrode (separated by a cation exchange membrane). Each working electrode chamber housed pH and dissolved oxygen (DO) probes, and was illuminated by two white fluorescent lamps to 29 W m^{-2} light intensity (measured at the electrode surface) simulating circadian conditions with 12h/12h illumination/dark periods with the temperature furthermore controlled at 24 ± 1 ^{o}C using fans.

The working electrodes of these cells were inoculated utilising a previously-enriched and electrochemically active seawater biofilm originating from the Sunshine Coast shore (eastern coast of Queensland, Australia) and fed continuously at 1.04 mL h^{-1} (hydraulic retention time (HRT) of 8 days) with filter-sterilised modified F2 medium. The pH in the

working electrode solutions (7.5 ± 0.2) was controlled as previously described (Darus et al. 2014). Inorganic carbon was supplied as 40 mM $NaHCO_3$ in the liquid feed, and/or by sparging a 3% CO_2/97% N_2 gas mix (rate 0.2 L min^{-1}) to condition the low/high Ci concentrations. DO depletion experiments were conducted by addition of 100 mM sodium dithionite into solution as a DO scavenger. Finally, the working electrodes were poised at oxidising conditions (+0.6 V vs. Standard Hydrogen Electrode (SHE)), to collect electrons derived from both high and low potential redox routes. For in-depth details of electrochemical cell construction, material/apparatus specifications, solution contents in working and counter electrode chambers and operation conditions, please refer to Darus et al. (2015).

5.2.2 Data collection

The rate of extracellular electron transfer to the working electrode was recorded as electrical current vs. time (chronoamperometry) by a multichannel potentiostat and reported as current density normalised to the projected surface area of the working electrode (24 cm^2). For DO depletion experiments, anodic current profiles were provided after subtraction of the abiotic control current from the biotic current to account for the effect of abiotic dithionite oxidation.

DO concentration was measured in solution in the working electrode chamber as described in Darus et al. (2014). Dissolved organic carbon (DOC) (measured as non-purgeable organic carbon (NPOC)) and Ci (measured as Total inorganic carbon (TIC)) were determined with a Shimadzu TOC-L CSH Total Organic Carbon Analyser with TNM-L TN unit, after being filtered with a 0.22 µm PTFE membrane.

For microbial identification, biofilm samples were scraped from the whole working electrode surface on the last day of experimental work. 100 mg of each collected sample were delivered to the Australian Centre for Ecogenomics (ACE) at the University of Queensland for genomic DNA extraction using the Maxwell® 16 Research Instrument (Promega) according to the manufacturer's protocol, with the Maxwell 16 Tissue DNA Kit (Promega) and further 16S-amplicon-pyrosequencing by Illumina Miseq Platform using 926F and 1392wR primer sets (Engelbrektson et al. 2010). Amplicon sequencing data analysis was conducted through application of the ACE mitag pipeline (http://wiki.ecogenomic.org/doku.php?id=amplicon_pipeline_readme). Representative

OTU sequences were BLASTed against the reference database (Greengenes version 2013/05 for 16S). Only OTUs with relative abundance above 1% were considered.

5.3 Results and discussion

5.3.1 Electrical response to illumination/dark phases

After inoculation, the photosynthetic consortia rapidly colonised the graphite-felt working electrodes by forming biofilms. EET from two photosynthetic microbial biofilms was recorded as anodic current. Initial conditions included a continuous supply of $NaHCO_3$ and CO_2 as Ci sources. As shown in Figure 5.1, anodic currents were observed during the dark phase from 5 days after inoculation. Moreover, anodic currents were not observed during illumination; instead, cathodic currents developed from day 10 onwards. Both anodic and cathodic currents increased over time, while no such reversible current profiles were observed in the electrochemical cell with abiotic aerated medium, confirming the biological nature of the phenomenon. Different metabolic processes must have caused the different electrical responses during the dark and illumination phases.

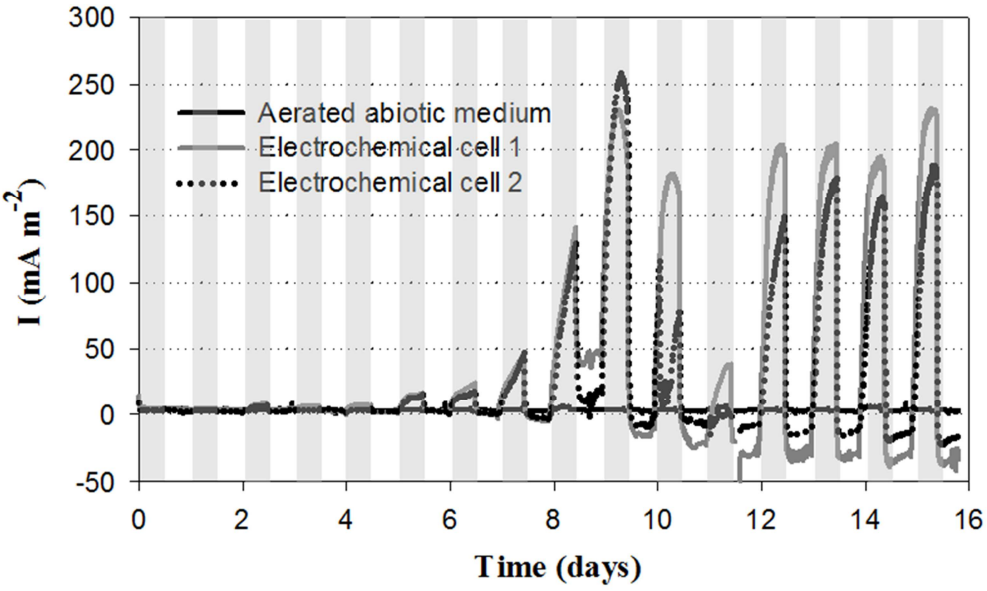


Figure 5.1 Profile of anodic/cathodic current in two identical three-electrode photo-electrochemical cells (electrochemical cells 1 and 2) containing previously-enriched seawater photosynthetic microbial consortium. An abiotic electrochemical cell containing continuously aerated medium was used as control. Interruptions to profile (day 10-12) were due to technical error. The shadowed and clear areas indicate 12 h dark and 12 h illumination phases, respectively

5.3.2 Microbial community

Analysis of the 16S rDNA of the biofilm attached to the electrode surfaces showed that the consortium was dominated by cyanobacteria (49% *Geitlerinema* and 3% *Lyngbya*) and heterotrophs composed of gamma-proteobacteria (8% *Amphritea* and 6% *Congregibacter*) and Bacteroidetes (13%), with minor presence of alpha-proteobacteria (12%), Planctomycetes of the Phycisphaeraceae family (5%) and other bacteria with abundance <1% (4% of total) (Figure 5.2). No significant abundance of eukaryotes was indicated.

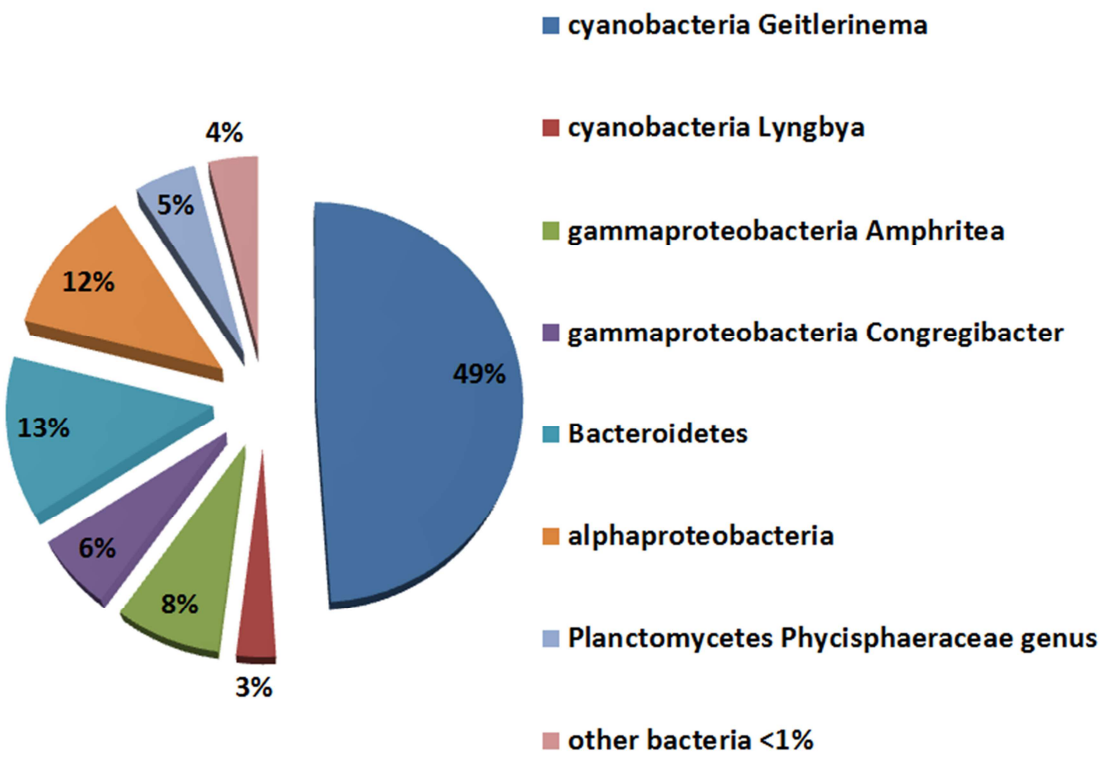


Figure 5.2 Sequence analysis of ribosomal 16S rDNA of bacterial community in biofilm attached to working electrode surface

The observed anodic current during the dark phase was likely due to anaerobic respiration using the organic carbon sources generated from CO_2 reduction (Tanaka et al. 1988), as this was the only electron donor available during the night and may be used by heterotrophic bacteria through oxidation of soluble organic carbon (*e.g.* from biomass degradation) and cyanobacteria via oxidation of stored organic carbon (Yagishita et al. 1993). The cathodic current observed during illumination was previously shown to be the result of cathodic oxygen reduction and correlated to the presence of gamma-proteobacteria (Darus et al. 2015; Rothballer et al. 2014) (see Graphical abstract).

5.3.3 Stored organic carbon enables anodic respiration during dark phase

It is known that the Ci uptake in cyanobacterial cells occurs over both the CO_2 uptake and the bicarbonate transporter systems (Rae et al. 2011). To investigate the effect of Ci availability on anodic EET, the gas mix used for sparging the reactor was cyclically switched to N_2 only. During these phases, the sole source of inorganic carbon was therefore the $NaHCO_3$ present in the feed, which was greatly reduced by the stripping of CO_2 by the N_2 stream: with $NaHCO_3$ as sole source of Ci (see days 1-16 and days 35-48, Figure 5.3A), the Ci concentrations measured at the end of dark time at days 9-15 and days 35-42 were in a range of 13-27 mg C L^{-1} (equivalent to 1-2 mM HCO_3^-) (Figure 5.3B) *i.e.* low Ci conditions. In the presence of CO_2 and $NaHCO_3$ (see days 20-34), the maximum measured Ci was 297 mg C L^{-1} (25 mM HCO_3^-) at day 34, thus considered as high Ci levels.

Figure 5.3 shows the decreasing rate of dark phase EET from photosynthetic microbial cell to electrode upon switching from high to low Ci conditions, and conversely an increase upon reverting to high Ci. Despite of fluctuating values, it is clear that the Ci tended to increase from low concentrations of 25 mg C L^{-1} at day 15 (just before CO_2 supply was initiated) to high concentrations of 297 mg C L^{-1} at day 34, in concomitance to the increase in anodic EET rates in the dark phase. When the Ci decreased from a high level of 297 mg C L^{-1} at day 34 (the day before CO_2 supply was stopped) to low level of 13 mg C L^{-1} at day 42, the anodic EET rate in the dark phase decreased.

The DOC in solution was measured at the end of dark time between day 9 and 42 (see Figure 5.3 and 5.S1, Supplementary material) to investigate any links to the dark phase-anodic current. The DOC concentrations were relatively constant in the dark regardless the Ci availability, with 9.6 ± 1.4 and 8.9 ± 1.8 mg C L^{-1} for electrochemical cells 1 and 2, respectively (Figure 5.S1), likely representing the constant rate of biomass decay, therefore hinting that the DOC was not a main electron source for the observed increase/decrease of the dark phase-anodic current.

These fluctuations of night time anodic currents can thereby only be explained by the effect of Ci on the rate of photosynthesis, and thereby the availability of organic carbon for dark-phase anodic respiration: the higher the inorganic carbon concentration, the higher the organic carbon accumulation by the cells and thus the higher the dark-phase extracellular electron transfer. Lacking an oxygen supply during the night time, it appears

that the photosynthetic consortium resorted to extracellular electron transfer for respiration and to avoid excessive intracellular accumulation of organic matter.

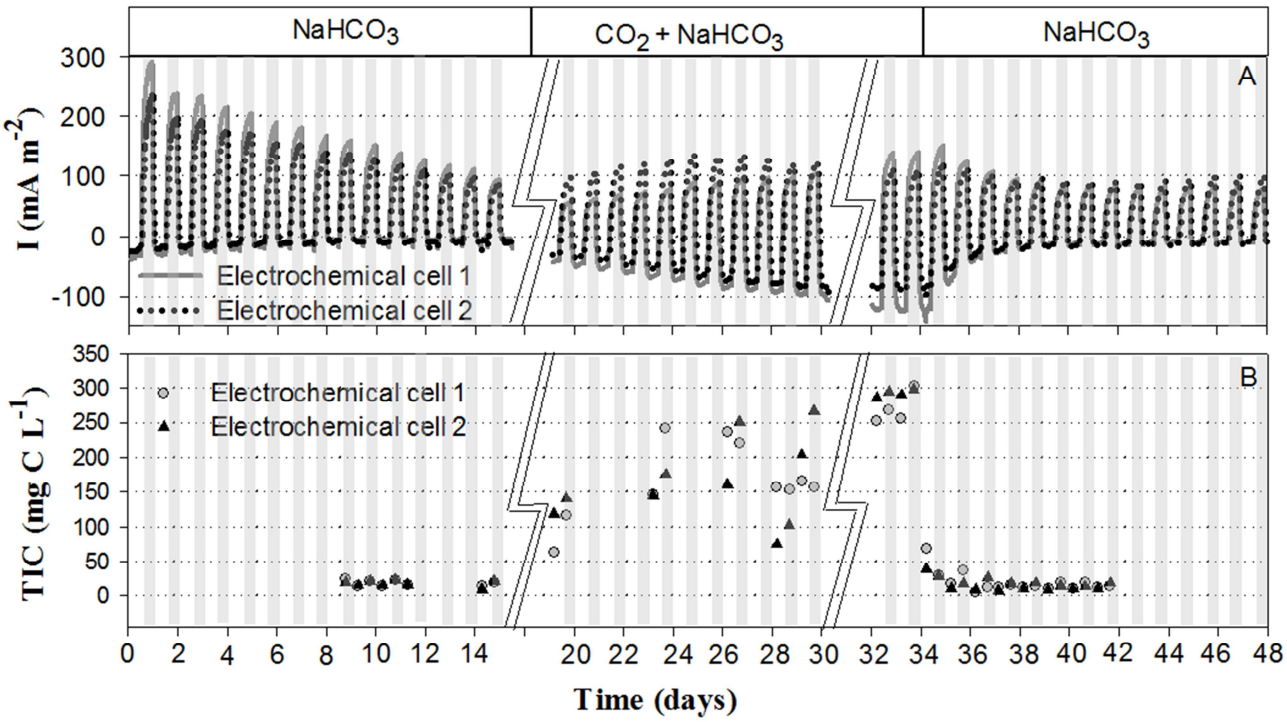


Figure 5.3 A. Electrical current profile of two identical electrochemical cells in the presence/absence of $NaHCO_3$ and CO_2 as Ci sources. B. Total Ci (measured as TIC) in the electrolyte of the working electrode chamber of the same electrochemical cells. Break lines indicate time intervals without data collection

The profiles of dissolved oxygen corroborate this hypothesis (see Figure 5.4). The effect of Ci availability on oxygen-evolving PS2 activity has long been debated. Importantly, Koroidov et al. (2014) recently reported that HCO_3^- acted as acceptor for protons from water splitting, and found that the switch from high to low Ci reduced the PS2 activity by up to 20%. In our work, a DO increase is related to a higher rate of O_2 evolution by PS2 – indicating a higher rate of intracellular electron transfer in the photosynthetic electron-transport-chain (Badger and Schreiber 1993) – and is in line with the rates of dark phase EET (Figure 5.4). The increase of this intracellular electron transfer rate likely promoted a higher assimilation of CO_2 into organic carbon (Burnap et al. 2013) in the form of intracellularly stored organic carbon and new cells/biomass (Badura et al. 2011; Yehezkeli et al. 2012). Hence, the increasing trend of dark phase-anodic EETs shown as anodic current in Figure 5.4 and 5.3A were likely the result of higher availability of stored organics (from fixed CO_2) as electron donors for dark phase respiration by cyanobacteria. This

hypothesis is supported by previous findings (Yagishita et al. 1993) showing a strong connection between stored glycogen concentration – an important form of stored organic carbon in cyanobacteria (Lindberg et al. 2010; Suzuki et al. 2010) – and the anodic current in dark phase, indicating the usage of stored glycogen as primary electron donor in the dark. The correlation between intracellular electron flow in the photosynthetic electron transport chain and Ci availability was previously proven (Zhang et al. 2012), whereby a low Ci condition (*e.g.* ambient CO_2 levels) was found to limit the rate of intracellular electron flow to the artificial soluble electron acceptor 2,6-Dimethylbenzoquinone (DMBQ).

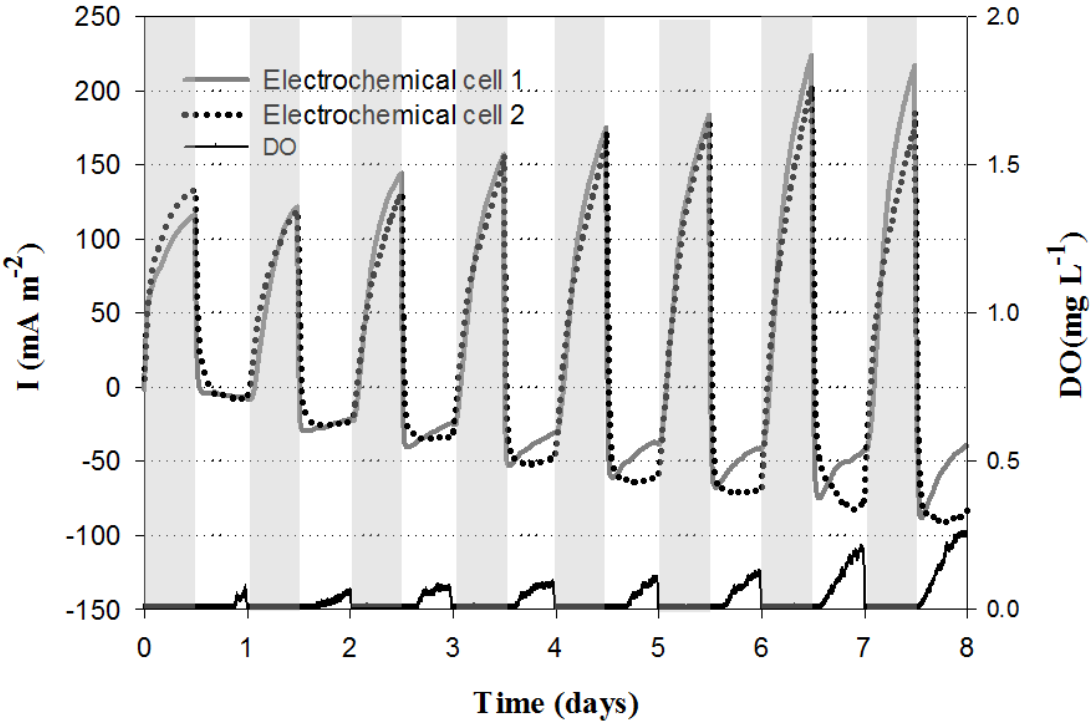


Figure 5.4 Profiles of DO measured in electrochemical cell 2 and electrical current recorderd in two identical electrochemical cells during high Ci condition

In addition, Figure 5.4 shows that DO was present and its increase was matched by a corresponding increase of maximum level of cathodic current. This effect was previously shown to be due to oxygen reduction at the electrode surface (Darus et al. 2015). There appears to be an anomaly in the daytime current profiles for Electrochemical cell 1, whereby the cathodic current peaks at the beginning of the day and then gradually decreases during the day. This may be a transient effect due to the possible temporary accumulation of organic matter within the biofilm, which may lead to increased chemo-heterotrophic activity during illumination. The transient effect may also be explained by a lower quantity of DOC during the day than night (see Figure 5.S1). Under light attenuation

condition in the lower layer of the biofilm *e.g.* when the cell density was high, the increasing oxygen concentration during the day (see Figure 5.4) may boost bacterial respiration.

5.3.4 Electrodes are used as electron sinks under reductive stress

The effect of Ci availability on anodic current generation was further investigated in the absence of both CO_2 and $NaHCO_3$. For comparative purposes, the experiments were started in low Ci condition with $NaHCO_3$ as the only Ci source. After steady-state levels of dark-phase anodic-current and illumination-phase cathodic current were attained, Ci-free conditions were imposed by replacing the electrolyte with a medium that did not contain any form of inorganic carbon. Although CO_2 can be produced through DOC degradation, continuous sparging of N_2 gas maintained the TIC to below detection limits (data not shown). Interestingly, following this step, anodic current production (instead of cathodic) was observed during illumination as shown in Figure 5.5 and its duplicate in Figure 5.S2. Prior to this change, anodic current was never observed under illumination with low or high Ci conditions (see Figure 5.3A and 5.4). To understand this phenomenon, the DO was measured at the end of the illumination phases and showed a decrease from 0.45-0.55 g L^{-1} in low Ci conditions (see profile a in Figure 5.5) to a level below detection limit in Ci-free conditions (see profile b in Figure 5.5).

The switch from high to low Ci condition was previously observed to increase internal electron transfer rate to DO (photoreduction) in PS1 from 20% to 60% (Allahverdiyeva et al. 2011). Besides photoreduction in PS1, a certain amount of oxygen can be also photoreduced in PS2 (Shimakawa et al. 2015) or reduced extracellularly by reduced redox compounds excreted by the microbial cells (see Darus et al. 2014 and a review in McCormick et al. 2015). Accordingly, the DO consumption by reduced redox compounds, together with a decrease of DO evolving-PS2 activity (Koroidov et al. 2014) might explain the absence of measurable DO in solution at Ci free conditions.

It is well established that light inactivates the glucose-6-phosphate dehydrogenase (Mann 2002; Stanier and Cohen-Bazire 1977; Tanaka et al. 1985), meaning that respiration of stored organic carbon by cyanobacteria is not responsible for the emergence of anodic current during illumination. This means that the anodic current observed was the result of electrons extracellularly diverted from the photosynthetic bacteria, either directly towards the electrode (Gorby et al. 2006; Sekar et al. 2014) and/or indirectly via intermediate

electron carriers *e.g.* H_2 (Olson and Maier 2002; Rosenbaum et al. 2005). The presence of intermediate compounds may lead to syntrophic processes, whereby H_2 or redox compounds excreted by photosynthetic bacteria would be subsequently oxidised by anode respiring bacteria. We therefore hypothesise that beside heterotrophic respiration of stored organic carbon (revealed by night-time anodic current), the day-time electrons are likely generated directly by photosynthetic water splitting: lacking their natural electron acceptor CO_2, these cyanobacteria are able to utilise the electrode as an electron sink for the photosynthetic electron transport chain. The absence of DO in solution lead to a steeper increase of anodic current upon switching from illumination to dark phase, as there is less oxygen competing with the electrode for extracellular electrons (in the form of redox compounds).

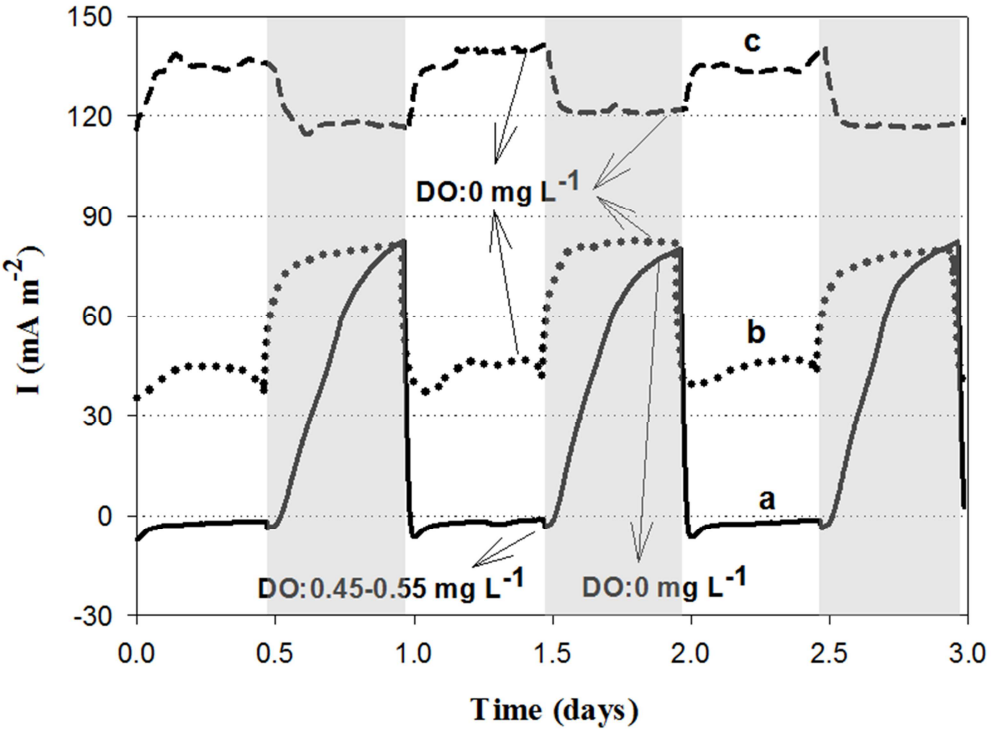


Figure 5.5 Steady state profile of EET and DO in electrochemical cell 2: a. low Ci conditions ($NaHCO_3$ as sole Ci source); b. Ci free condition; c. Ci- and oxygen-free conditions using a DO scavenger. Arrows represent the time when DO was measured in all illumination/dark phases

The occurrence of day-time anodic EET was nevertheless not a complete proof that these electrons originated at the photosynthetic electron transport chain, since they could also be the result of the oxidation of organic matter by heterotrophic bacteria. To exclude the latter possibility, an oxygen scavenger was added to eliminate any trace of DO in the

system. After removing the competing electron sink (dissolved oxygen) from the biofilm (see profile c in Figure 5.5 and 5.S2), more electrons were diverted towards the electrode during the illumination than during the dark. If the anodic current was the result of heterotrophic respiratory processes only, it would have not been higher during the illumination than the dark. This observation strongly supports our hypothesis that electrons generated during illumination by PS2 are diverted onto an extracellular insoluble electrode, which functions as terminal acceptor, thus enabling continuous electron flow even if Ci is absent.

The maximum levels of dark-phase anodic-currents were relatively unchanged upon the switch from low to free Ci condition (Figure 5.5 and 5.S2). This may seem counterintuitive and may reflect the cyanobacterial endogenous respiration – sustained by the cycling of carbon within the biofilm (inorganic to organic during illumination and vice versa during dark phases).

In conclusion, this electron diversion onto an extracellular insoluble electrode has not been described before and may be a natural way for cyanobacteria and other phototrophic bacteria to maintain redox balance for survival under reductive stress which may arise from low Ci concentrations or high light intensity. In natural environments, available extracellular electron acceptors may include mainly dissolved oxygen, but also mineral oxides and elemental sulphur. Information on the reasons behind this EET feature is important to achieve long-term application such as harvesting the electrons for electricity. Further work to quantify electron flow is essential to allow more insight on the magnitude of the anodic current for the cell activity.

5.4 Acknowledgements

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The authors declare no conflict of interest.

5.5 Supplementary materials

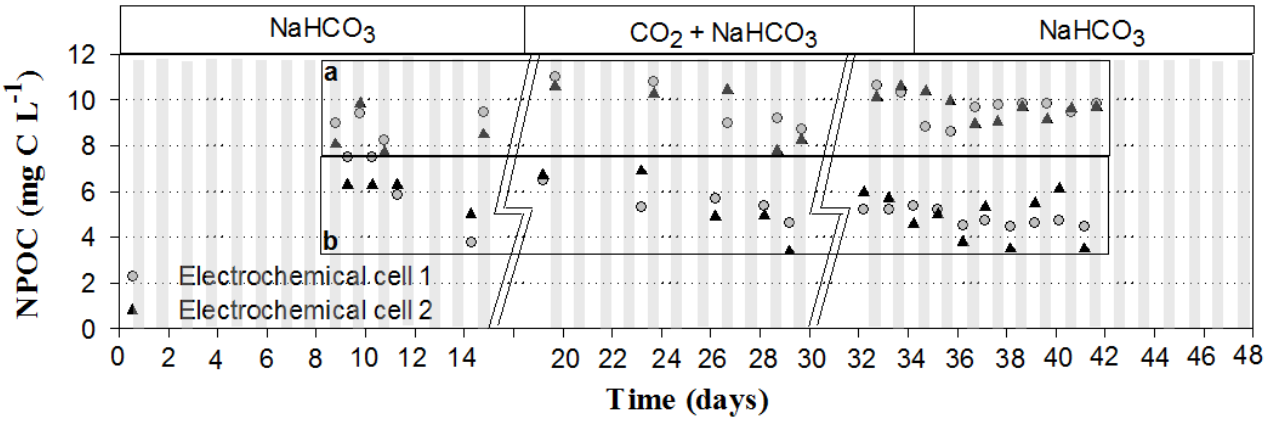

Figure 5.S1 Dissolved organic compounds (DOC) measured as non purgeable organic carbon (NPOC) in solution of working electrode chamber of two identical electrochemical cells: a. in the dark; b. under illumination. Break lines indicate time intervals without data collection

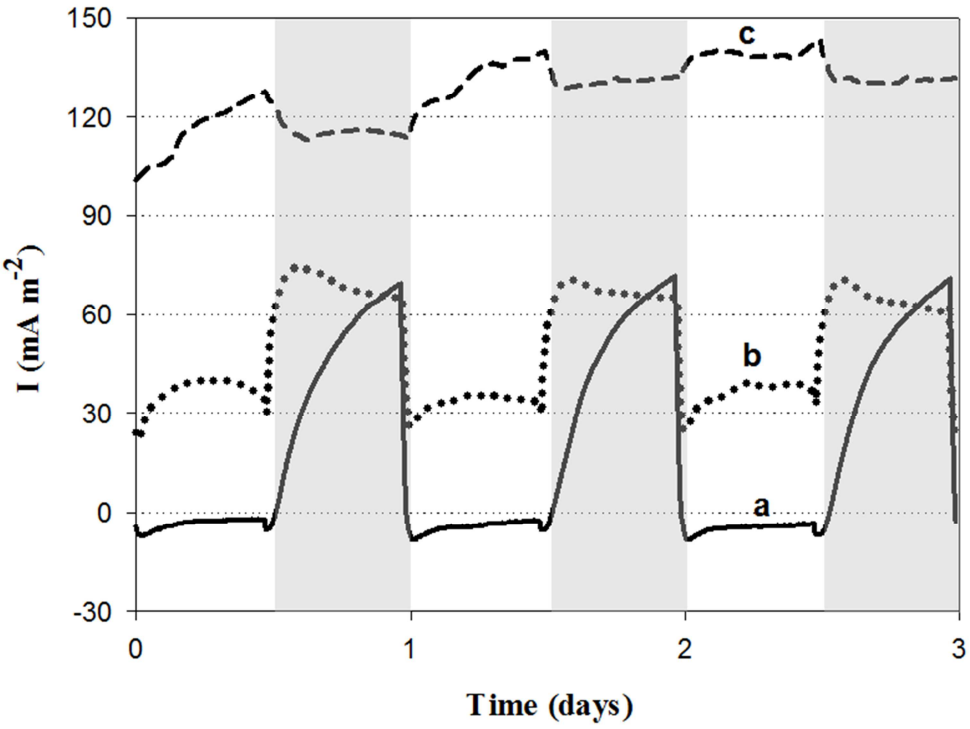

Figure 5.S2 Steady state profile of EET in electrochemical cell 1: a. low Ci conditions ($NaHCO_3$ as sole Ci source); b. Ci-free conditions; c. Ci- and DO-free conditions

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Chapter 6

Redox polymers enable day/night photo-driven electricity generation by mixed seawater consortia

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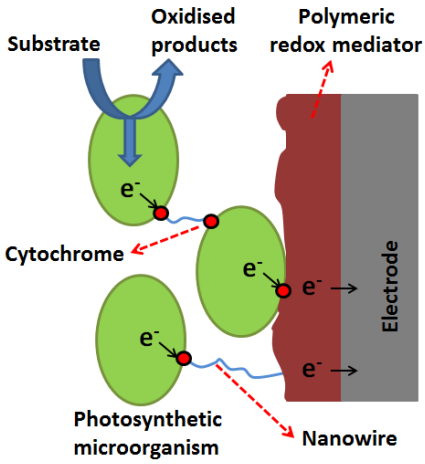
Biophotovoltaic devices modified with immobilised polymeric osmium/azine redox-mediators exhibited a considerable electrical output enhancement (64/43-fold under light/dark conditions, respectively). More importantly, the systems exhibited uninterrupted current generation at same magnitude levels during day/night cycles, paving the way towards solar energy conversion bio-panels that will not require energy storage peripherals.

Graphical abstract



Highlights

- High current densities improvement on biophotovoltaic system (up to 60-fold).
- Uninterrupted day/night current generation.
- Repeatable, stable performance for over 20 day/night cycles of operation.

Keywords: Azine redox mediator, Electrochemical polymerisation, Photoelectrochemical cell, Photosynthetic microbial consortia, Polymeric osmium complex

6.1 Introduction

Biophotovoltaic systems (BPVs) are an emerging biotechnology for the light-driven renewable production of electricity. BPVs rely fundamentally on the ability of various photosynthetic microorganisms, primarily cyanobacteria, to divert electrons from certain metabolic pathways – the exact routes remain a matter of debate (McCormick et al. 2015) – onto insoluble electron acceptors *i.e.* solid electrodes. To date however, low electrical outputs and polarity reversal during illumination remain strong limitations towards any real-world practical implementation of BPVs (He et al. 2009; Strik et al. 2010; Darus et al. 2015b). Previous research efforts have focused on maximising current output by utilising soluble electron mediators which allow for electron transfer from the enzymatic active sites within the cells to the electrodes (Delaney et al. 1984; Park and Zeikus 2000; Rosenbaum et al. 2010; Tsujimura et al. 2001; Nishio et al. 2013). Nevertheless, the use of soluble mediators requires continuous replacement, which makes their use unsustainable, costly and in some cases even harmful to the natural environment (Rosenbaum et al. 2010; Patil et al. 2012). Recent investigations in the field of bioelectronics have demonstrated that electron shuttling via immobilised redox mediators such as polymeric osmium complexes

(Osadebe and Leech 2014; Yuan et al. 2016) and polymeric azines (Bauldreay and Archer 1983; Karyakin et al. 1999; Yang et al. 1998) (e.g. polymethylene blue (PMB), polythionine or polymethylene green (PMG)) can be a stable and efficient alternative (see review in Du et al. (2014). Indeed, by immobilising polymeric osmium complexes onto working electrodes, the biocatalytic performance of pure cultures of the cyanobacterium *Leptolyngbia* (Hasan et al. 2014) and the *microalga Paulschulzia* (Hasan et al. 2015) have been significantly improved.

For the practical implementation of BPVs however, the use of a microbial consortium is considered advantageous over axenic cultures, because consortia typically produce higher electrical outputs and have higher resilience to variable physicochemical and process conditions (McCormick et al. 2015). In this study, we report for the first time on the significant enhancement of current output by a mat-building marine photosynthetic consortium dominated by cyanobacteria, through immobilisation of polymerised osmium complexes or azine mediators onto photoelectrochemical cell anodes. The electrode modifications resulted not only in significant output enhancement, but also in the disappearance of current reversals, leading to uninterrupted day/night anodic current production at similar magnitude levels.

6.2 Experimental Section

6.2.1 Setup preparations

The experiments were conducted in 3-pin photoelectrochemical cells of 70 mL volume with plain or modified graphite rods (diameter 4.73 mm) as working electrodes. For each cell, the side of the graphite rod was covered with a rubber sheath, leaving only the base as working surface (area: 17.6 mm^2), whilst a Ti wire (length 4 cm, diameter 0.5 mm) and a KCl-saturated Ag/AgCl electrode (+0.197 V *vs* SHE) were used as counter and reference electrodes, respectively. An electrogenic photosynthetic marine biofilm dominated by cyanobacteria (52%) with the rest consisting mainly of heterotrophs (48%) was scraped off a working photoelectrochemical cell (see enrichment process and full community composition in Darus et al. (2015a) and used as inoculum. The electrochemical cells were operated inside a dark box in batch mode, fed with filter-sterilised modified F2 medium (Darus et al. 2015b) at pH 7.8 ± 2 containing 40 mM $NaHCO_3$ (as sole carbon source and pH buffer) and illuminated by white LED lights at 29 W m^{-2} in circadian cycles of 4 h day/4 h night. The experiments were maintained at 24 ± 1 ^oC inside the box using a fan.

6.2.2 Electrode modifications

Prior to modification, the bases of the graphite rods were polished with 1 µm alumina powder for 3 min, rinsed and sonicated with distilled water. The polymeric osmium complex [Os(2,2'-bipyridine)$_2$(polyvinyl-imidazole)$_{10}$Cl]Cl (hereafter referred to as Os-1) and polymeric osmium complex [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(polyvinyl-imidazole)$_{10}$Cl]Cl (hereafter referred to as Os-2) were prepared according to previous reports by Ohara et al. (1993) and Osadebe and Leech (2014), respectively. Individually, 25 µL solution of 7 mg/mL polymeric osmium complex (in distilled water) was mixed with 5 µL solution of 7 mg/mL poly (ethylene glycol) diglycidyl ether (PEG-DGE) (Sigma-Aldrich). 5 µL of the mixture was applied evenly on the base of the graphite rod, dried for 12 h at room temperature and cured for another 24 h at 8 ^{o}C.

For the azine redox mediators, 1 mM monomer solutions of methylene green (MG), methylene blue (MB) (Tokyo Chemical Industry Corp) and thionine (Sigma-Aldrich) were prepared individually by dissolving in 100 mM PBS buffer of pH 7.0 and 100 mM $NaNO_3$. The monomer electro-polymerization was then carried out by immersion of the graphite rods working surfaces in the monomer solution and undertaking cyclic voltammetry (CV) from -0.2 to +1.4 V *vs* SHE for 10 cycles at 10 mV s^{-1}, followed by immersion in distilled water for ≥3 h to remove any residual monomer.

6.2.3 Evaluations

The surface modification was confirmed by cyclic voltammetry (CV), conducted between 0 and +0.8 V *vs* SHE at a scan rate of 10 mV s^{-1} in modified F2 medium, with a multichannel potentiostat (CHI1000B, CH Instruments, USA). Moreover, the electrochemical performance of biotic modified anodes, and abiotic and biotic unmodified controls, was evaluated by chronoamperometry (CA) until steady-state was observed (approx. 5 days or 15 light/dark cycles) and for a further 9 cycles (for the duplicate reactors), which is the data hereby presented. CA experiments were run on all electrochemical cells at a poised anode potential of +0.6 V *vs* SHE to maximise electron extraction from photosynthetic and respiratory electron transport chains. All experiments were run in duplicate independent electrochemical cells.

All experiments were run in duplicate independent electrochemical cells.

6.3 Results and discussion

To confirm the role of the consortium on current generation, the responses of abiotic and biotic unmodified anodes were first investigated via CA. As shown in Fig. 6.1, no current oscillation was observed for the abiotic unmodified anode. Following inoculation, two cells with unmodified electrodes produced a small amount of current, which was higher at night (9 and 6 mA m^{-2} respectively) than during the day (5 mA m^{-2} for both). This day/night pattern has previously been observed with other photosynthetic microbial consortia (Darus et al. 2014; He et al. 2009) and explained by the detrimental effect of photosynthetically-evolved oxygen during illumination (Darus et al. 2014) and the higher rates of anodic respiration (of organics produced from fixed CO_2 during the day) in the dark.

To enhance the extracellular electron transfer (EET) of the microbial consortium, various redox polymers were immobilised onto the anode surfaces, with CVs subsequently conducted to confirm that the modifications were successful. Redox peaks were observed for both polymeric osmium complexes (Fig. 6.2A), whereas increased anodic and cathodic currents across the scan range were observed with polymeric azine mediators (Fig. 6.2B), confirming the presence of electroactive material on the working electrode surfaces.

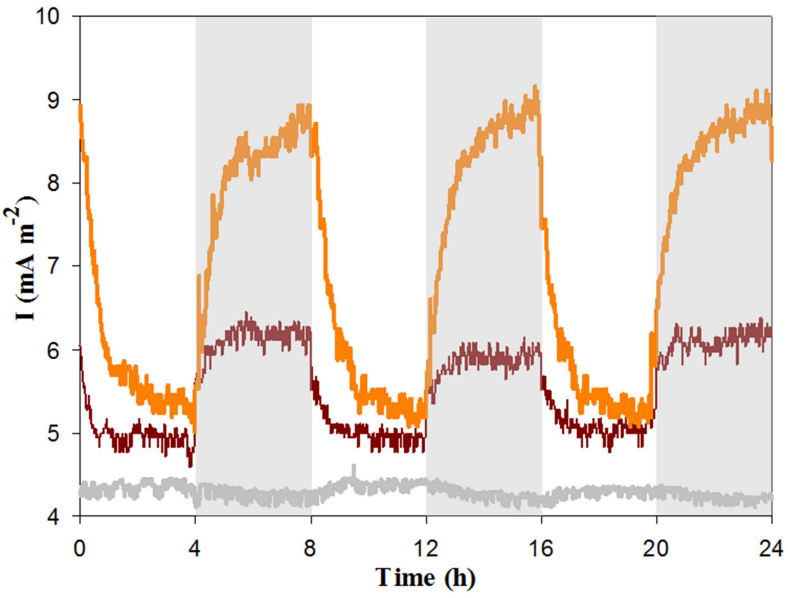
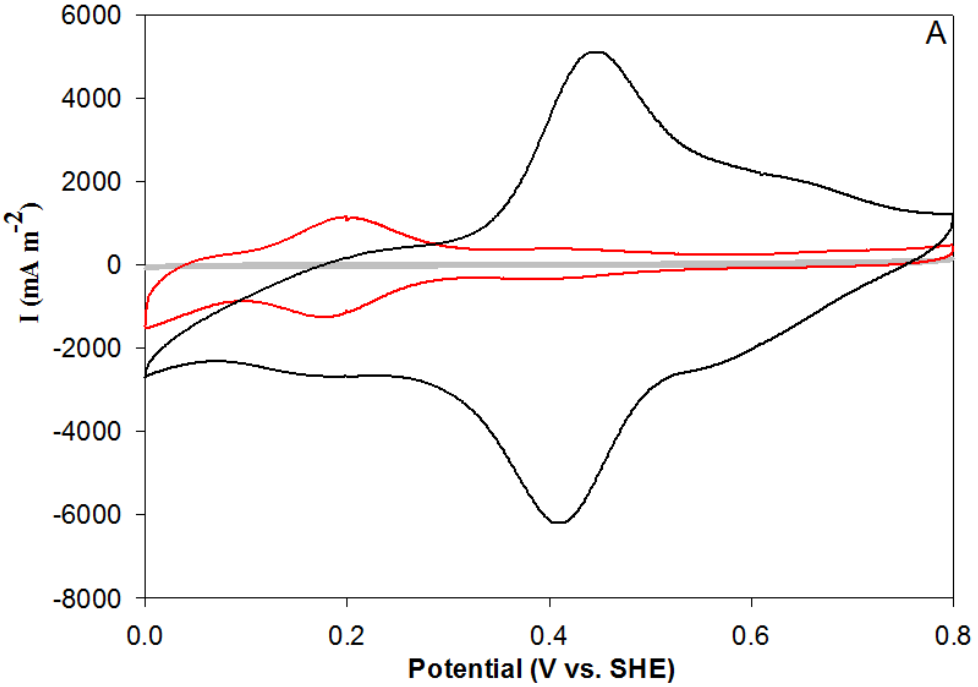


Figure 6.1 Chronoamperometric anodic current profile of unmodified electrochemical cells: abiotic (grey), first biotic reactor (orange) and duplicate biotic reactor (maroon)

With the help of these modifications, and as shown in Fig. 6.3 and Fig. 6.S1 for the duplicate reactors (see Supporting Information), the anodic current output increased during both day and night when the redox polymers were immobilised on the anodes. Under

illumination, the enhancement was 64 ± 6, 19 ± 5, 21 ± 2, 22 ± 0 and 20 ± 2 times, respectively for Os-1, Os-2, polythionine, PMB and PMG, while during the night the improvement was lower but still 43 ± 8, 13 ± 6, 14 ± 5, 15 ± 4 and 13 ± 3 times respectively. Interestingly, the use of these polymeric mediators in both repetitions led to slightly higher anodic currents during the day than at night (see Fig. 6.3 and 6.S1), which is a reversal of the behaviour previously observed without mediators (see Fig. 6.1). This phenomenon suggests that photosynthetic microorganisms are able to use extracellular anodes as terminal electron acceptors for the photosynthetic metabolism (Darus et al. 2015a).

The better performance of the redox-polymer-bearing anodes compared to the controls may be attributed to the formation of three-dimensional multi-layers of electroactive material, maximising the electrical wiring between the biocatalysts and their terminal electron acceptor (Bauldreay and Archer 1983; MacAodha et al. 2012; Murthy and Reddy 1983).



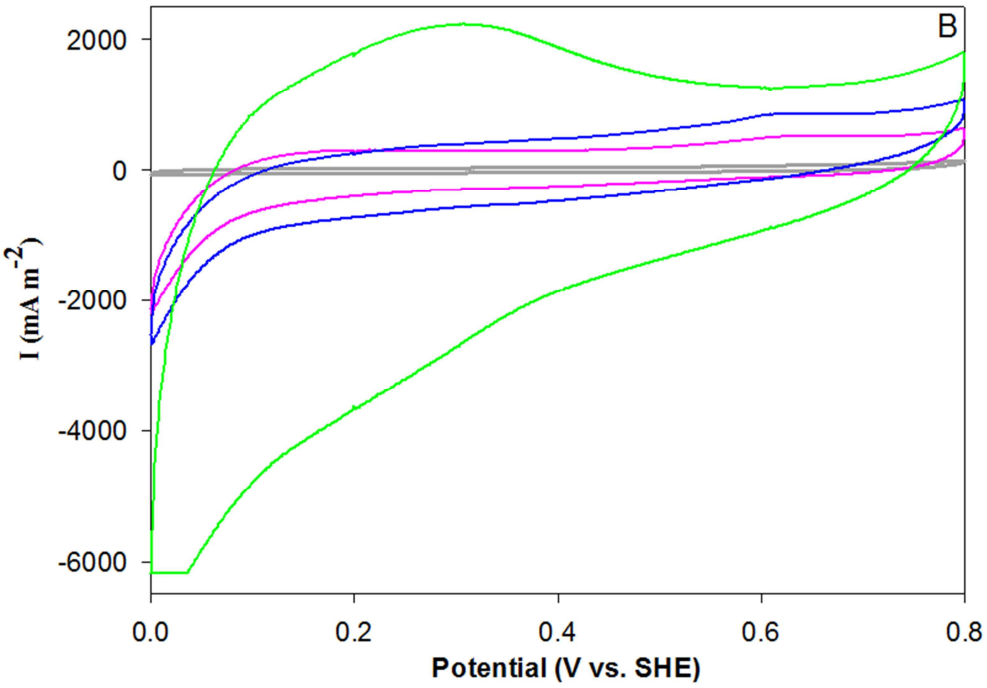


Figure 6.2 Anodic cyclic voltammograms with immobilised (A) Os-1 (black) and Os-2 (red); (B) polymeric azine redox mediators – polythionine (pink), PMB (blue) and PMG (green), compared to unmodified anode (grey) in the absence of biocatalyst

With regards to the polymeric osmium complexes, a considerably higher current generation was observed with Os-1, reaching 320 ± 28 mA m^{-2} under illumination and 317 ± 29 mA m^{-2} in the dark, whilst Os-2 systems reached only 93 ± 23 mA m^{-2} and 89 ± 20 mA m^{-2} for day/night conditions respectively. These significant differences may be explained by the higher redox potential of Os-1 (+0.428 V vs. SHE) *versus* Os-2 (+0.188 V *vs* SHE; see Fig. 6.2), which may (*i*) speed up electron harvesting from the photosynthetic electron transport chain (Hasan et al. 2015) and/or *(ii)* lead to a lower likelihood for Os-1 to get re-oxidised by photosynthetically-evolved oxygen, a problem that severely inhibits EET, as previously demonstrated (Darus et al. 2014). Similar effects were previously reported for pure cyanobacterial (Hasan et al. 2014) and algal species (Hasan et al. 2015). Moreover, although the deposition methods and amounts of Os-mediator utilised were identical, it is also possible that *(iii)* different concentrations of Os-1 and Os-2 were ultimately immobilised onto the studied electrode surfaces (as hinted by the CVs in Fig. 6.2). Therefore, the performance differences could be attributed to the combination of all three factors.

Nevertheless, the biological cultures – which had been electrochemically active for years without polymers in other reactors (Darus et al. 2015b; Darus et al. 2015a) – responded immediately and stably (see Fig. 6.S3 for longer-term performance) to the redox-polymer additions with consistent differences in electrical output throughout the length of the experiments, indicating lack of any toxicity effects and demonstrating the stability of the modifications over time (as previously demonstrated for >6 months in physiological solutions (Tsujimura et al. 2014).

The sustained day/night anodic current in all modified anodes was furthermore confirmed by CV conducted in day and night conditions (after reaching steady-state). As shown in Fig. 6.S2, at a potential +0.6 V *vs* SHE, the biotic day/night anodic current output was higher than the abiotic one for all modified anodes. Moreover, at this potential the day-time anodic current was higher than at night. These clear (albeit not massive) differences of biocatalytic current further confirm the biological nature of current generation in these biophotovoltaic systems (as shown in Fig. 6.3).

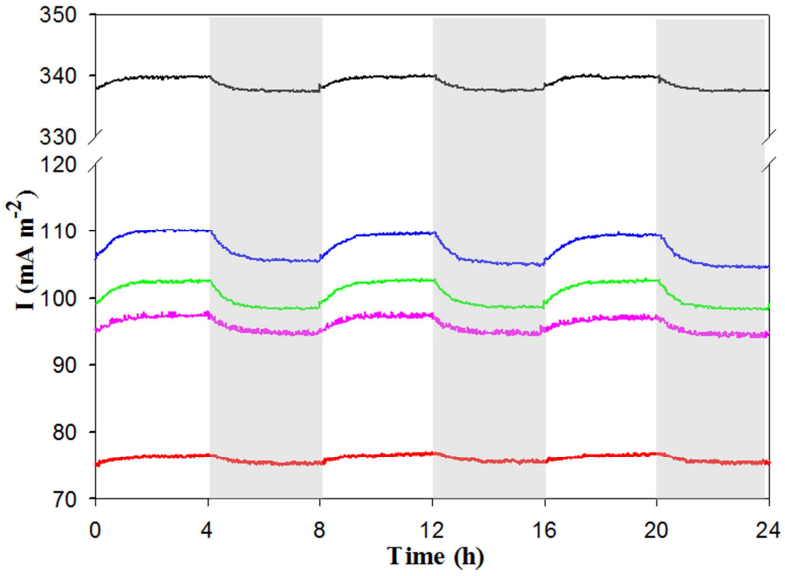


Figure 6.3 Chronoamperometric anodic current profile of first modified electrochemical cells: Os-1 (black), Os-2 (red), polythionine (pink), PMB (blue) and PMG (green) in presence of the biological consortium

6.4 Conclusions

Electrochemical testing indicates the chosen polymeric mediators were successfully immobilised onto anode surfaces and significantly enhanced light-driven electron transfer

from microorganisms to electrodes. The co-immobilisation of these polymeric mediators and photosynthetic consortia as biocatalysts led to significant current enhancement and uninterrupted anodic current generation during day/night cycles, an important step towards the implementation of electricity production by biophotovoltaic systems in niche contexts.

6.5 Acknowledgments

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6.6 Supplementary figures

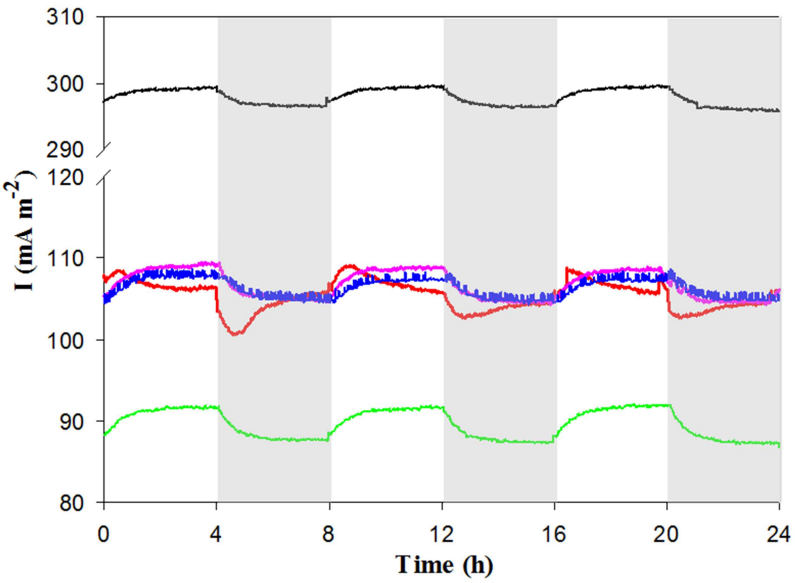

Figure 6.S1 Chronoamperometric anodic current profile of duplicate modified electrochemical cells: Os-1 (black), Os-2 (red), polythionine (pink), PMB (blue) and PMG (green) in presence of the biological consortium

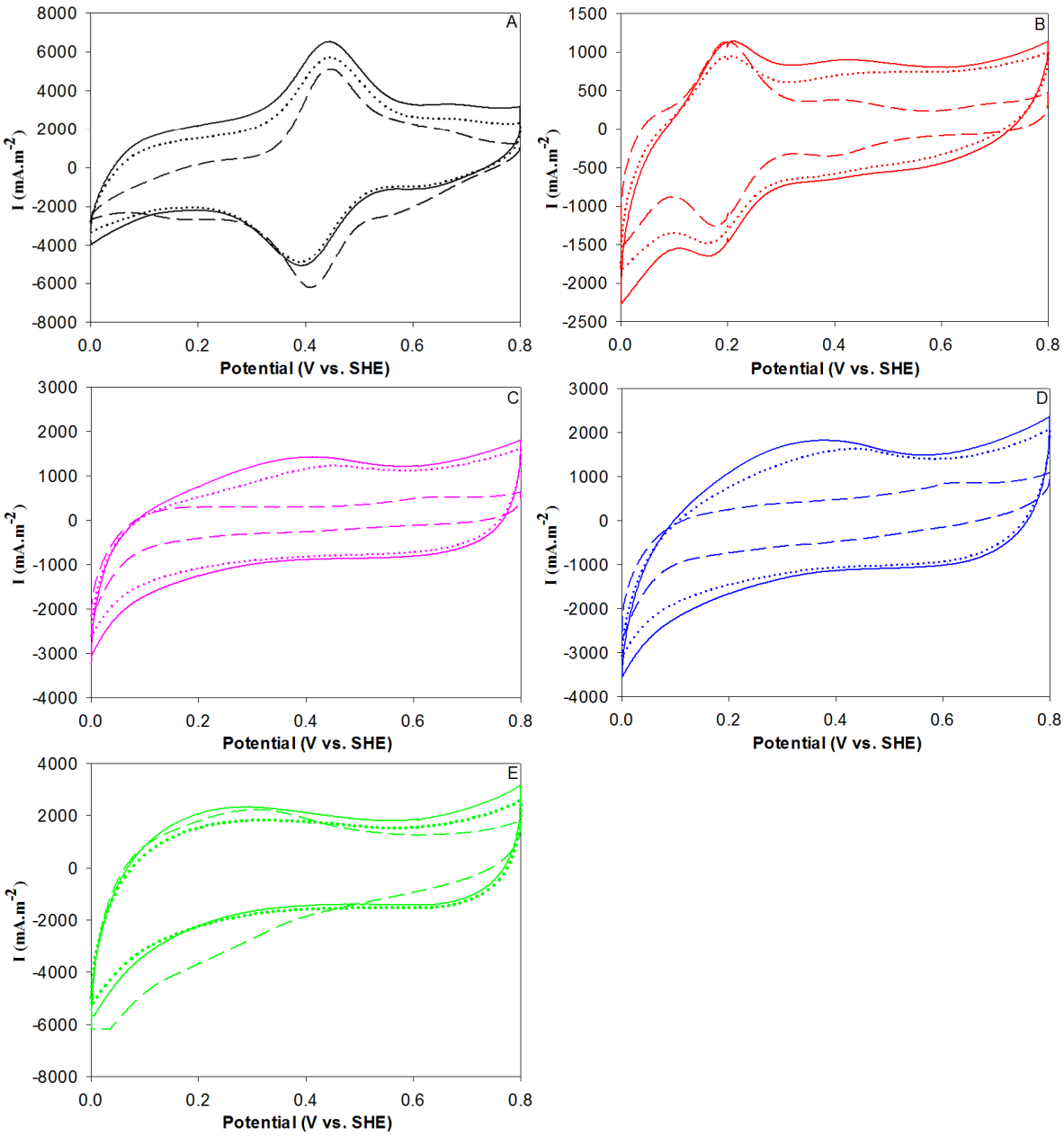

Figure 6.S2 Anodic cyclic voltammograms with immobilised polymers: (A) Os-1; (B) Os-2, (C) polythionine, (D) PMB and (E) PMG, in the absence (long-dashed-lines) and presence of the biological consortium: day (solid lines) and night (dotted lines)

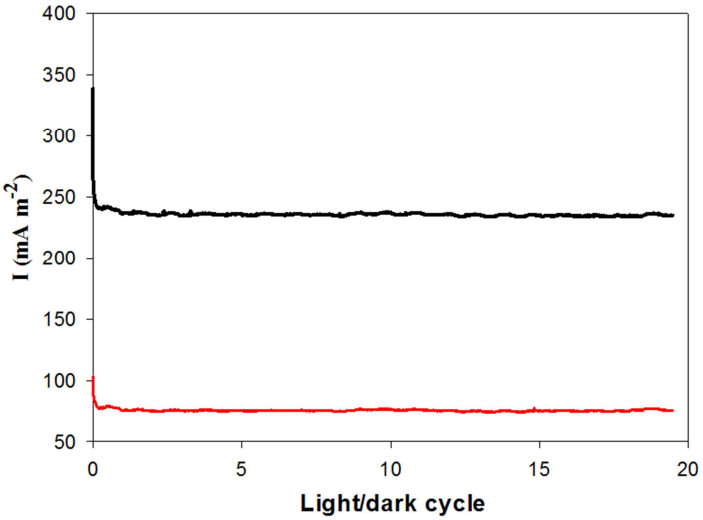

Figure 6.S3 Anodic chronoamperometry profiles of two modified electrochemical cells: Os-1 (black), Os-2 (red) over 19 day/night cycles (4h/4h)

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Chapter 7
Conclusions, outlook and future research

In this chapter the insights gained over the PhD candidature are summarised and discussed. Ideas are also put forward for further research towards possible applications of this technology.

7.1 Conclusions

The overarching goal of this thesis was to understand the natural occurrence of phototrophic microorganism with EET capability and capture their biocatalytic capability to create a device for the conversion of sunlight to electricity. We found that electron flux to electrodes is not a widespread feature in environmental photosynthetic culture and most likely affected by microbial community composition. The EET seems however to be only ubiquitous in seawater environments, therefore limiting the applicability of MSC devices to seawater environments. Moreover, photosynthetic evolved oxygen detrimentally affects the photo anodic-EET in fresh and seawater cultures, by competing with the anode as electron acceptor and/or its use for oxygen reduction, consequently decreasing the capture of electrons at the anode.

Under illumination and without added organic carbon, the flux of electrons from cyanobacterial consortia to the anode was driven by light via electron generation by water oxidation, possibly with hydrogen as intermediate electron carrier. Despite the presence of photosynthetic evolved oxygen that extremely inhibits hydrogenase activity, hydrogen may be generated via catalytic reaction of the nitrogenase enzyme. The EET occurred even in the absence of inorganic carbon, which suggests that this metabolic feature may be a mechanism for survival in stressing reductive condition. Under darkness, the flux of electrons was found to be attributed to the consumption of organic matter. This outcome provides insight towards physiological and metabolic reasons for the observed EET.

Importantly, the previously observed detrimental effect of oxygen to photo current generation did not appear (i.e. a higher day time-anodic current was exhibited than at night) when redox polymers (e.g. polymeric osmium complexes and polymeric azine mediators: polymethylene blue and green, polythionine) were co-immobilised with mat-building seawater photosynthetic consortia dominated by cyanobacteria onto anodes. Additionally, this co-immobilisation led to a continuous anodic current generation during the day/night cycles. Compared to soluble mediators, redox polymers avoid the need of an ion exchange membrane to prevent mediator redox cycling between anode and cathode. The maximum improvement of anodic current outputs was achieved with electrodes immobilised by polymeric osmium complexes [Os(2,2'-bipyridine)$_2$(polyvinyl-imidazole)$_{10}$Cl]Cl, to 320 ± 28 mA m^{-2} under illumination and 317 ± 29 mA m^{-2} during the dark phase.

7.2 Outlook and future research

The state of the art power and current density of this tool is still very low even with the enhanced system, and certainly very far from existing photovoltaic solar panel (40 A m^{-2}). Another issue is that the mediators selected in this work have high redox potential and this is not suitable for power producing devices because low potential anodes are needed when oxygen reduction is the cathodic process, which even catalytically proceeds at high rate only below +0.4 V vs. SHE (Clauwaert et al. 2007).

Further experimental work is needed to integrate the new modified bioanodes with effective oxygen-reducing cathodes to create a working MSC device. Research is needed to overcome the detrimental effect of oxygen, for example by introducing mediator-immobilised electrodes that have high resistance to oxygen re-oxidation and at the same time are capable of speeding up electron harvesting from the PETC. Future research will also need to deal with long term operation, to solve issues such as competition by non-electroactive organisms, removal of excess biomass, minimising potential losses, etc. The use of almost freely available resources such as natural medium (*e.g.* sea water), nutrient from wastewater effluents (nitrogen and phosphorus) and inorganic carbon source from CO_2-containing gas effluent (waste reuse/removal), in combination with production of valuable products and biomass would provide added benefits.

The future applications of MSCs are promising, for examples to power small devices in remote areas, and even more importantly as biosensors. Malik et al. (2009) estimates the potential of photosynthetic consortial-driven MSC to achieve a maximal output of 20 A m^{-2}, or a power of 3 W m^{-2}. These outputs, although remarkable for a biological solar panel, will not be competitive with existing photovoltaic technologies. However, new niches might arise such as the use of these MSC devices for biosensing. Of especially high impact would be the use of these biosolar sensors for the in-situ detection of cyanobacteria for the early detection and prevention of cyanobacterial blooms.

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Curriculum Vitae

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Awards

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