Characterisation of novel electroactive consortia from environmental samples enriched through potentiostatic growth

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SINGAPORE

**CHARACTERISATION OF
NOVEL ELECTROACTIVE CONSORTIA FROM
ENVIRONMENTAL SAMPLES ENRICHED
THROUGH POTENTIOSTATIC GROWTH**

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Singapore Centre for Environmental Life Sciences Engineering
(SCELSE)**

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A thesis submitted to the Nanyang Technological University in partial
fulfilment of the requirement for the degree of
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2017

“The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music.”
~Lewis Thomas

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CHARACTERISATION OF NOVEL ELECTROACTIVE CONSORTIA FROM ENVIRONMENTAL SAMPLES ENRICHED THROUGH POTENTIOSTATIC GROWTH

Summary

Despite the great microbial diversity nature has to offer, a relatively small group of electrochemically-active microorganisms (EAM) has been discovered. This work aimed to explore the use of a three-electrode set-up to enrich and isolate novel EAM. The approach was chosen as an alternative to the conventionally used microbial fuel cell. A three-electrode set-up using a carbon felt working electrode was developed and optimised with a pure culture of *Pseudomonas aeruginosa*, a species not conventionally tested using this type of system. The platform was sensitive enough to detect current generation using phenazines as redox mediator. Furthermore, simultaneous, real-time electrochemical detection of the redox-active metabolites *Pseudomonas* quinolone signal and pyocyanin was achieved in live cultures using voltammetry, with implications for diagnostic devices in healthcare settings.

After this validation, the platform was then used to characterise a complex microbial community, during long-term electrochemical enrichment. Tropical climate sediment was chosen as inoculum source, as it is currently under-explored in this context which frequently focuses on enrichment from waste water. Specifically, sediments from a canal system and a mangrove region of Singapore were chosen and enriched separately for EAM. Comparison revealed the enriched community from canal sediment was superior to that from the mangrove sediment in terms of electrogenic activity when evaluated by chronoamperometry, and was

chosen for further characterisation. Voltamperometric techniques and electrochemical impedance spectroscopy, performed over a wide range of potentials, dynamically characterised extracellular electron transfer (EET) over the four month enrichment. Metagenomics and metatranscriptomics analysis revealed that *Geobacter metallireducens* was heavily enriched on the electrodes, a species not typically associated with a lactate-fed system such as this. *Chlorobium*, *Clostridium* and *Rhodopseudomonas* were also abundant, indicating tropical climate sediments are host to a range of microorganisms capable of EET.

Two novel isolates were grown in pure culture from the enrichment, identified to be from the genera *Enterobacter* and *Aeromonas*. Both isolates were capable of EET in defined media on carbon felt and screen-printed electrodes. Both could avail of acetate and lactate as electron donor. Additionally, the *Enterobacter* isolate could perform mediated electron transfer using the soluble redox shuttle 2-hydroxy-1,4-naphthoquinone (HNQ), indicating it may have availed of this mode of EET during the initial enrichment.

This work outlines a comprehensive methodology for characterising and isolating novel EAM from unconventional inocula. It also highlights the applicability of this set-up for monitoring a wide range of EAM, in both mixed communities and pure culture. Specific genera characterised range from high current generators such as *Geobacter* to more modest performers such as *Pseudomonas*, *Enterobacter* and *Aeromonas*. In addition to characterising EAM, such technology can be used for biosensors, as demonstrated by the detection of redox-active metabolites of the pathogen *Pseudomonas aeruginosa*.

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Author Publications & Patents During Candidature

Journal Articles

Doyle, L. E.; Marsili, E., Methods for enrichment of novel electrochemically-active microorganisms. *Bioresource technology* **2015**, *195*, 273-282.

Seviour, T. W.*; **Doyle, L. E.***; Lauw, S. J. L.; Hinks, J.; Rice, S.; Nesati, V.; Webster, R. D.; Kjellenberg, S.; Marsili, E., Voltammetric profiling of redox-active metabolites expressed by *Pseudomonas aeruginosa* for diagnostic purposes. *Chemical Communications* **2015**, *51* (18), 3789-92.

***Co-first author**

Artyushkova, K.; Roizman, D.; Santoro, C.; **Doyle, L. E.**; Fatima Mohidin, A.; Atanassov, P.; Marsili, E., Anodic biofilms as the interphase for electroactive bacterial growth on carbon veil. *Biointerphases* **2016**, *11* (3), art. 031013.

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Meetings Attended

1. 2nd Water Research Conference. 20-23 January 2013. Singapore.
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4. 228th Electrochemical Society Meeting. 11-15 October 2015. Phoenix,
Arizona [oral presentation].

List of Abbreviations

ARB	Anode-respiring bacteria
BES	Bioelectrochemical system
CA	Chronoamperometry
CV	Cyclic voltammetry
DET	Direct electron transfer
DPV	Differential pulse voltammetry
EAM	Electrochemically-active microorganisms
EC	Electrochemical cell
EET	Extracellular electron transfer
EIS	Electrochemical impedance spectroscopy
HNQ	2-hydroxy-1,4-naphthoquinone
MEC	Microbial electrolysis cell
MET	Mediated electron transfer
MFC	Microbial fuel cell
R_{ct}	Resistance to charge transfer

Chapter 1: Introduction

This chapter has been re-formatted from a review article that was published in Bioresource Technology ¹, of which Lucinda Elizabeth Doyle was first author.

1.1 Electrochemically-active microorganisms

Electrochemically-active microorganisms (EAM) are organisms capable of exchanging electrons with insoluble electron acceptors, such as metal oxides in nature or electrodes in the laboratory, via extracellular electron transfer (EET) ¹⁻³. Known by many names (electricigens, exoelectrogens, anode-respiring bacteria), EAM are relevant to metal and sediment biogeochemistry as they are frequently found in nature as dissimilatory metal reducers ⁴⁻⁵. EAM interface with the electrode in several ways, and the reader is referred to recent reviews on this topic ⁶⁻⁹. So far two distinct mechanisms of EET have been described; direct electron transfer (DET), which involves microbial nanowires and outer membrane-associated c-type cytochromes, and mediated electron transfer (MET), which involves soluble electron shuttles. While largely defined in terms of their ability to pass electrons to a solid external electron acceptor, some EAM have been shown to receive electrons from the electrode, using it as an electron donor ¹⁰⁻¹². This latter ability has found application in bioelectrosynthesis, where electrons are delivered

¹ Doyle, L. E.; Marsili, E., Methods for enrichment of novel electrochemically-active microorganisms. *Bioresource Technology* **2015**, 195, 273-282.

from the cathode and reduce organic and inorganic compounds to form electrobiocommodities (reviewed by Lovley and Nevin¹³).

EAM are usually found in anaerobic subsurface environments, where the low redox potential and limited nutrient concentrations facilitate the emergence of metal-reducing metabolic pathways. However, EAM can be found in oxygen-limited environments. It is important to note the presence of aerobic members in the community can be beneficial in terms of removing O₂ which may diffuse into the local environment, thus inhibiting EET and/or negatively impacting obligately anaerobic microorganisms. Finally, some of the most common strains, conventionally not known for their EET abilities, can behave like EAM under a well-defined set of environmental conditions, e.g., self-produced phenazines by *Pseudomonas aeruginosa* and other self-generated mediators by *Escherichia coli* can act as electron shuttles to reduce electrodes¹⁴⁻¹⁵. An electroactive community is defined by the presence of at least one EAM strain which can exchange electrons with a solid terminal electron acceptor. Mixed communities of strict anaerobes, fermenters and EAM are likely to be the norm in nutrient-rich environments, either natural or man-made; as reflected in the recent shift from pure culture studies to mixed consortia studies.

The unusual metabolic abilities of EAM have been harnessed to produce current, with the first example dating to more than 100 years ago¹⁶. The engineered system to this purpose is termed microbial fuel cell (MFC), a combination of a microbial anode and a microbial/chemical cathode, which is today considered an example of a two chamber bioelectrochemical system (BES). Fundamentally in a single chamber BES, either an oxidative or reductive current

can be produced. In the case of the former, EAM are grown on an electrode at positive potential (using an anode) and oxidise the nutrient in solution, generating an oxidative current, with the electrode as terminal electron acceptor. Alternatively, when microorganisms are grown at negative potential (using a cathode), the electrode acts as electron donor, resulting in a reducing current. In the case of MFCs, the small power output generated by the potential difference between anode and cathode can be used to power independent sensors ¹⁷⁻¹⁸ and small autonomous systems (reviewed by ¹⁹). Some of the most interesting biotechnological applications of EAM are in waste water treatment and bioremediation, where the removal of organic carbon or other contaminants is associated with a small power output ²⁰. Despite intensive research, MFCs have not yet reached full-scale application and the power output of such devices has not surpassed a few W/m² in real-world applications. The reader is referred to Oliveira et al. ²¹ for an updated review on full-scale MFCs and their applications.

Microbial electrolysis cells (MECs) are a modification of BES technology, and necessitate the application of an overpotential to achieve thermodynamically-unfavourable reactions. Such devices facilitate bioelectrosynthesis of organic compounds and show good promise for green chemistry and desalination, provided some of the scalability issues are resolved ²². A key point when considering EAM is that electrode potential, as opposed to soluble electron acceptor concentration (e.g. O₂), dictates the thermodynamic energy available for the microbes to grow ²³, hence the application of such an overpotential may be necessary for EAM growth and enrichment from diverse inocula.

In addition to engineered devices, EAM are also relevant to microbially-induced corrosion (MIC) in fresh water and, to a larger degree, seawater ³. Live biofilms have been shown to influence corrosion rates ²⁴, and members belonging to the genus *Shewanella*, among others, have been found to play a beneficial role in MIC inhibition (MICI), as reviewed by Mansfeld ²⁵. Hence characterisation of charge transport chains in EAM may allow the design of mitigating strategies for MIC.

To date, few EAM have been discovered, with much of the research exploring the nature of EET focusing on two iron-reducing genera isolated from sediments: *Shewanella* spp., facultative anaerobes that thrive in the high potential region of the iron reduction zone ²⁶⁻²⁸, and *Geobacter* spp., strict anaerobes that predominate at the lower iron reduction potential ²⁹⁻³⁰. In addition to increasing our fundamental knowledge of EET mechanisms, new strains may hasten the application of EAM in waste water treatment, thus providing vast savings to waste water treatment plants (WWTPs), where aeration currently accounts for 30-55% of total energy required ³¹. However, this and other envisioned applications of EET call for a variety of EAM superior to those characterised to date, in terms of their ability to work at high/low potentials and resistance to organic contaminants. Furthermore, it is now recognised that in contrast to single strains thriving under controlled laboratory conditions, EAM likely function in a consortium in natural environments and thus should do so in technological applications which generally involve a broad variety of organic substrates and mixed waste streams. As such, there is great interest in the discovery of novel EAM strains and consortia, leading to a variety of

enrichment techniques to isolate these specialised organisms from their microbially-rich natural environments.

There are at least five major shortcomings in the quest for novel EAM. They concern the inoculum source, the growth medium used, the reactor configuration, the electrochemical characterisation and the community profiling conducted. The starting inoculum in many studies is frequently waste water, owing to EAM's envisioned application in energy recovery from waste water treatment. Current methods for enrichment of novel EAM rely mostly on conventional microbiological methods, whereby sediments are gradually enriched for the organism of interest under iron-reducing conditions in defined medium. Additionally, characterisation of electroactivity typically relies on simplified electrochemical methods, such as power output on anode potential over time, which do not provide sufficient information on the abilities of the EAM community. This chapter will demonstrate where these shortcomings originated and why they have not been sufficiently addressed. We will then discuss key advancements toward the mitigation of these limitations, as seen in recent technical literature. Finally, some future perspectives will be provided of how bioprospecting of EAM will evolve in the near future and what results this endeavour may yield.

1.2 Starting material

The source of inoculum is of great significance in determining the outcome of an enrichment procedure, as the inoculum harbours the entirety of diversity from which the EAM will emerge. Here some recent EAM enrichment studies are reviewed in terms of the starting microbial material.

1.2.1 Sediments and soils

Owing to sediment and soil's diverse microbial populations and wealth of redox mediators such as humic acids and terminal electron acceptors such as metals, this type of material is frequently chosen as the starting point for EAM studies. Furthermore, microorganisms in such environments are likely to be associated with solid particles, thus providing a selective pressure for the development of extracellular respiration via EET.

Salvin, Roos and Robin ³² explored tropical mangrove sediments as MFC inoculum, and via electrochemical analysis identified it for the first time as a promising starting material from which to generate current (12 A.m^{-2}), using acetate as carbon (C) source. In a separate study, Nguyen et al. ³³ chose tropical mud samples for an EAM enrichment in MFCs at low resistance with Fe(II) as the only electron donor. *Pseudomonas teesidea* was the dominant species in the community, with current likely produced via the microbially-produced redox mediators, phenazines. Such findings underline the importance of anaerobic environments to drive the evolution of bacteria capable of utilising electron acceptors other than oxygen. Indeed Miceli et al. ³⁴ conducted a study on anaerobic inocula for enrichment in an MEC, with samples including a variety of soils and sediments. Pyrosequencing revealed only some of the samples were highly abundant in *Geobacter* post-enrichment, with unknown community members also thriving, highlighting the benefits of expanding our choices of inoculum.

Freshwater and marine sediments have also been explored, e.g. Song et al. used various freshwater sediment samples in glucose-fed single-chamber MFCs, and found variations in Fe(III) concentrations had a significant effect on potential

difference across electrodes and on the anode community structure, as determined via 16S rRNA gene profiling³⁵. The community consisted of many non-EAM, in addition to the familiar electrogens *Shewanella* and *Geobacter*. However, no novel EAM species were identified in this long-term enrichment (>100 days). While the previous study was anodic enrichments, Pisciotta et al. used marine sediments as starting material in a MFC setup, before switching to potentiostatic mode to enrich for electron-consuming organisms in a cathodic study, with community analysis revealing microorganisms similar to *Eubacterium limosum* (*Butyribacterium methylotrophicum*), *Desulfovibrio* sp. A2, *Rhodococcus opacus*, and *Gemmata obscuriglobus*³⁶. However, further research is needed to determine the electroactivity of these putative cathode-oxidising microorganisms.

In a comparative study, Dunaj et al.³⁷ found agricultural soil to be superior to forest soil in terms of power generation, with notable differences in their respective anodic communities also apparent. Their study found that high power generation gave rise to less diversity, with agricultural anodes dominated by *Deltaproteobacteria*, *Geobacter*, and to a lesser degree, *Clostridia*, while low-power MFC anode communities were dominated by *Clostridia* alone.

Enrichment of EAM from rice paddy soils was carried out in an MFC³⁸. The combination of plant and root microbial communities resulted in high current production under both light and dark conditions. Electrophoresis showed enrichment of bacteria related to *Rhizobiales*. Recently, enrichment from lagoon sediment slurry showed a repeatable shift in the microbial anodic community when examined across a range of electropositive potentials³⁹.

All these studies point in the direction of high EAM diversity in soils and sediments, highlighting this inoculum source as a viable starting point in the search for novel species.

1.2.2 Waste water

Many studies (see Table 1.1) conducted on EAM have used waste water as an inoculum, due to its easy availability, high reproducibility and remarkable diversity inherent in the communities of waste water treatment plants (WWTPs) ⁴⁰. However, waste water harbours mostly mesophilic microorganisms adapted to circumneutral pH. Considering that insoluble minerals or metals, which may serve as external electron acceptors, are generally absent from typical municipal WWTPs, one must consider that any EAM found in such locations may be incidental in their electrogenic abilities, as opposed to having evolved specifically to carry out EET. This is in contrast to environments where such a function would afford the bacterium an advantage, such as a bioremediation or MIC site.

As a typical WWTP is comprised of a number of different stages (e.g. primary clarifier, activated sludge basin, secondary clarifier, anaerobic digester) ⁴¹, some studies have adopted the approach of combining inocula from different sections of the plant. Hou, Sun and Hu ⁴² used a mixture of anaerobic and aerobic sludge to determine the effect of adding glucose and Congo red dye either simultaneously or sequentially on dye-decolourisation and electricity generation. They reported that dye-decolourisation was independent of enrichment style but current generation was improved with simultaneous addition of the two components, with *Geobacter* notably only present in the reactor operated in this enrichment style, as observed by 16S rRNA gene profiling. In a study addressing

the importance of electrode material, Larrosa-Guerrero et al.⁴³ used a mixture of brewery waste water diluted in domestic waste water to investigate the effect of anode material in open and closed circuit MFCs, finding communities were only influenced by electrode material at open circuit.

Even more unusual sources of waste have been reported, with Rismani-Yazdi et al.⁴⁴ using bovine rumen contents as inoculum for an MFC examining the effects of electrical resistance on bacterial diversity, concluding that a different consortium was enriched with each resistance value and finding evidence that fermentation dominated over anaerobic respiration in MFCs operated at higher loads.

While useful in terms of evaluating how efficient EAM are at degrading waste water, the choice of such material as inoculum omits a large proportion of microbial diversity.

Table 1.1 Summary of recent enrichment studies employing community profiling approaches.

Reference	Inoculum Source	Enrichment Method	Electrode Material	Metagenomics	Metatranscriptomics	Dominant Microorganisms	Comments
35	Freshwater lake	MFC	Carbon cloth	Yes	No	<i>Geobacter</i> , <i>Shewanella</i> , <i>Geothrix</i> , <i>Firmicutes</i> , <i>Fibrobacteres</i>	While fermenters were found in both reactors sequenced, EAM were only detected in one.
45	Municipal waste water (primary clarifier)	MFC	Carbon cloth	Yes	Yes	<i>Desulfobulbaceae</i> and <i>Desulfuromonadales</i>	-
46	Rice paddy field sediment	MFC	Graphite felt	Yes	No	<i>Geobacteraceae</i>	Found that dominant <i>Geobacter</i> species depended on electron donor added.
47	Domestic waste water (primary clarifier)	MEC (Power-source controlled)	Graphite fibre brush	Yes	No	<i>Geobacter/Shewanella</i>	Used GeoChip technology.
48	Soil	MEC (Potentiostat-controlled)	Granular graphite	Yes	No	<i>Clostridium</i>	-

1.2.3 Extreme environments

Relatively few studies have been conducted using inocula from extreme environments. Extreme pH is particularly relevant for EET to metal ions, while EAM operative under extreme temperatures hold exciting promise for industrial application.

Fu et al.⁴⁹ operated a hyperthermophilic MFC inoculated with microbes originating from a high-temperature petroleum reservoir. Peak performance was seen at 95 °C and the community was dominated by *Caldanaerobacter subterraneus* and *Thermodesulfobacterium commune*.

Wang et al.⁵⁰ used a U-shaped MFC to degrade petroleum hydrocarbon-contaminated saline soil, finding hydrocarbon-degrading organisms in connected MFCs were 100 times more abundant than in open-circuit MFCs, and highlighting that such devices can be used to achieve specialised enrichments.

Pierra et al.⁵¹ reported the enrichment of two EAM strains, *Geoalkalibacter subterraneus* and *Desulfuromonas acetoxidans*, in saline sediments in potentiostat-controlled reactors operated at pH 7 and 5.5. Percentage abundance of individual strains ranged from 86% to 99% depending on reactor, highlighting the quality of selection that can be achieved under highly controlled conditions.

Although working with extremophiles is challenging, it is expected that such efforts will yield novel EAM strains and EET pathways, as well as novel redox enzymes of biotechnological relevance.

1.3 Medium composition

Currently there is no consensus about the ideal growth medium for EAM enrichment and it is not clear whether the medium composition is the critical factor in EAM enrichments. Acetate and lactate-rich media have largely been used for enrichment of *Geobacter* and *Shewanella*, respectively. Early enrichment experiments used uncharacterised growth media such as waste water to enrich EAM in MFCs⁵². This undefined enrichment selected a community that coupled chemical oxygen demand (COD) degradation with electricity production, but no comparison with a non-enriched community was reported. Following the early discovery that acetate-consuming bacteria were primarily involved in electricity production, acetate media were used for MFC enrichments. For example, Lee et al.⁵³ reported enrichment and community analysis via electrophoresis. However, no control experiment with a different electron donor/carbon source was reported. This enrichment method works well with any starting community, however over-reliance on it may hinder discovery of EAM in unusual inocula. It is important to note that, while limited in number, there are studies which have tested mixed carbon source media. One such study was reported by Kaur et al.⁵⁴, who used acetate, butyrate and propionate as the sole carbon source in the context of developing an MFC-type biosensor for detecting volatile fatty acids. The reader is referred to a comprehensive review by Pant et al.⁵⁵ detailing substrates used in MFCs.

Most media compositions to date have catered to anodic enrichments, with various carbon sources being explored. However, with the increased attention cathodic enrichment, a new range of electron donorless media with a variety of electron acceptors should be developed. While the production of

electrobiocommodities via MECs has gone some way towards addressing this, the use of even non industrially-relevant electron acceptors may yield novel EAM and/or novel EET mechanisms.

1.4 Reactor configuration

Reactor configuration in EAM research can take the form of MFCs, MECs and potentiostat-controlled three-electrode setups (i.e. half cells). The choice of reactor configuration should not be taken lightly in enrichment studies, as results vary widely depending on design, as will be discussed in this section.

1.4.1 Microbial fuel cells

As seen in Section [1.2](#), the majority of EAM studies have been conducted using MFCs; engineered devices designed to harness the flow of bacterial current and consisting of both an anode and a cathode ⁵⁶. The decoupling of results related to the EAM themselves and those related to the multiple components and configurations of the MFC can be cumbersome. Additionally, MFCs are designed primarily to maximise power output and as such are not optimised to probe the electroactivity of the communities present. In MFCs, the potential of the anode, at which the EET is taking place, is not controlled and is allowed to drift over the course of the experiment. This affords less control over enrichment conditions and may select primarily for EAM capable of adapting to a range of potentials. Srikanth and Mohan ⁵⁷ conducted a study investigating the effect of distance between the anode and cathode on the electrogenic capabilities of an MFC operated using waste water, finding that distance was inversely related to current generation, highlighting the non-bacterial considerations involved in the performance of such a system. Raman and Lan ⁵⁸ looked at the impact of both

electrode distance and anodic chamber stirring on performance in a photo MFC, a device which incorporates photosynthetic microorganisms. Their approach consisted of using the Tafel equation to determine reaction kinetics at different electrode distances.. However, it should be noted that to the best of the author's knowledge no reports have evaluated the differences in taxonomic composition of electrodes at different spacings. Pham et al.⁵⁹ employed a high-shear enrichment technique in a stacked MFC configuration in order to select for a strongly attached and dense biofilm. Their study indicated that high flow rates resulted in a more electroactive community than lower flow rates. Taking this study as an example, one can see that future work investigating this phenomenon could take a more streamlined approach by carefully controlling the applied potential with a potentiostat while altering flow rates. Such a study would not be possible in an MFC due to the confounding factor of the potential drift inherently seen at the anode.

Additionally, the community analyses of MFC studies cited in Table 1.1 generally revealed a shallow distribution slope in terms of community diversity at the conclusion of an enrichment, indicating a high level of enrichment had not occurred. The question of how to increase the steepness of these slopes needs to be addressed in order to isolate novel EAM and reconstruct their genomes after deep sequencing in metagenomics, thus enhancing reference genomes in the databases.

In summary, as MFCs are an application of EAM and not an ideal method for enrichment of novel EAM, potentiostatic methods are to be preferred from a fundamental research point of view. While potentiostats are admittedly costly pieces of equipment, controlled potential can also be achieved in high-throughput

power source-controlled reactors similar to the screening device described by Call and Logan ⁶⁰.

1.4.2 Microbial electrolysis cells

MECs differ from MFCs in that a potential is applied to the anode, thus supplying the system with energy in order to drive an otherwise thermodynamically unfavourable process in terms of electron donor and acceptor electrochemical potentials. Torres et al. ²³ investigated the effect of a range of different potentials on current density in a continuous flow MEC configuration, finding that lower anode potentials saw the enrichment of a more specialised community capable of higher current densities. This contrasts with the more diverse community which resided on the anodes poised at the universally attractive higher potentials, at the expense of current generation. As with Liu et al. ⁶¹, they found *Geobacter* to dominate the low-potential electrodes. Parameswaran et al. ⁶² utilised an MEC in order to study syntrophic interactions between ARB and non-ARB in a consortium enriched from a mixture of anaerobic and return activated sludge. Call and Logan ⁶³ used MEC to study *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA utilising lactate, extending the use of MECs beyond only enrichment studies.

While the use of MECs may be a step in the right direction in terms of controlling anode potential, we are still faced with a certain amount of confounding factors since these devices are primarily designed to produce electrobiocommodities. Issues such as non-standard reactor design can limit comparison between studies. Furthermore, the presence of both an anode and a cathode in the one experiment can make it difficult to draw conclusions about the

communities residing on each electrode, their response to external stimuli, and their shift with time.

1.4.3 Potentiostat-controlled bioelectrochemical cells

A potentiostat is a device that controls the potential of a working electrode (either anode or cathode). Potentiostatic experiments are typically conducted using a three-electrode setup consisting of a working electrode where the reaction of interest is occurring, a counter electrode which balances charge, and a reference electrode against which the potential is measured ⁶⁴. Compared with MFCs, it is a more streamlined approach which is useful in quantitatively characterising EAM and lends itself to applying numerous electrochemical techniques such as voltammetry to probe the underlying mechanisms of electron transfer. However the instrument's costliness has made it less widely used when compared with the relatively cheaper MFC technology.

Liu et al. ⁶¹ proposed a novel enrichment approach using this conventional three-electrode set-up. In agreement with previous findings in the literature, an electroactive biofilm was found to form on the working electrode. In order to enrich the consortium further, a sterile electrode was introduced to the set-up. This was then colonised via bacterial detachment from the existing electrode. This saw an increase in current density for the secondary biofilm with respect to the primary biofilm (500 $\mu\text{A}/\text{cm}^2$ versus 250 $\mu\text{A}/\text{cm}^2$), indicating successful enrichment. This is in contrast to the alternative approach of using sequential biofilm transfers in order to enrich EAM to increase current density ³⁴. The reasoning behind this particular technique is to provide a selective pressure in order to allow the EAM to dominate the electrode microbial community.

Potentiostatic half-cell enrichments serve as proof of concept for further industrial applications. Furthermore, novel species enriched therein may provide further insight into EET mechanisms which can be applied to previously-discovered EAM to increase efficiency.

1.5 Electrochemical analysis

Electroanalytical methods are relevant to EAM enrichment as they can be used to monitor current generation, pinpoint the temporal appearance of novel redox-mediators and the emergence of EET pathways, elucidate the effect of biofilm formation on conductivity, and ultimately depict the community shift from a diverse consortium to an efficient EAM enrichment. This section introduces some of the many techniques that can easily be used to characterise EAM across the timescale of an enrichment with the use of a potentiostat. It is important to note that electroanalysis can only provide limited information on the actual EET mechanism, as many microbially-produced redox-active compounds have very similar voltammetric signatures. Finally, the potential and current of electrochemically-active species in communities depend strongly on the electrode material and even on its history (e.g., biofouling and biofilm formation/thickness). Some of these issues can be resolved by analysing the cell-free supernatant on standard electrodes such as gold and glassy carbon.

1.5.1 Chronoamperometry

Chronoamperometry (CA) is a technique that continuously applies a precise potential to the working electrode while simultaneously measuring the resultant current output⁶⁴. In the case of EAM, the microorganisms will exchange electrons with this electrode and as such the choice of potential must be carefully

considered. In the case of anodic enrichments, the potential should be electropositive enough to ensure the transfer of electrons from the microbial cytochromes and/or shuttles is thermodynamically favorable, but not so high as to pose an oxidative stress to the cell. Many studies have opted to set the electrode at 200 mV vs Ag/AgCl. Other studies have chosen to apply a negative potential, thus providing a smaller energetic payoff to the microorganism (frequently *Geobacter* which thrives in the low-potential region). Alternatively a cathodic enrichment can be undertaken, whereby the electrode acts as electron donor thus should be poised at a sufficiently negative potential. In this case a negative current is recorded ³⁶. Notably, studies have shown that the application of controlled potential not only affects performance but can also have an impact at the taxonomic level, as determined through 16S rRNA gene profiling ^{39, 65}.

In addition to quantifying the electricity produced (anodic enrichments) or consumed (cathodic enrichments), CA can provide insight into the health of a culture since the current is closely related to cell growth ⁶⁶⁻⁶⁷. Indeed the growth stage of the culture can be roughly estimated visually, with the CA output (I vs t) mimicking that of a traditional growth curve, depicting lag, log, stationary and decline phases. However, this is correct only for early biofilm formation in short-term experiments, either in batch or continuous mode. For more long-term experiments, the CA output is affected by diffusional limitations and biofilm stratification, thus becoming a poor descriptor of the biofilm local electroactivity. This is especially true in enrichment networks containing mixed microbial consortia where the microbial population fluctuates with time ⁶⁸.

As a technique, CA is analogous to monitoring the power output of a traditional MFC, however it provides more of an insight into the abilities of the

electroactive community at a fundamental level, as opposed to the interplay between these abilities and the reactor configuration of the particular MFC in question. It is important to caution that CA alone is usually not sufficient to describe an enrichment satisfactorily. However, when combined with other techniques such as voltammetry and electrochemical impedance spectroscopy, a greater characterisation of a consortium's capabilities emerges.

1.5.2 Voltammetry

Voltammetry is a potentiostat-controlled electrochemical method which facilitates observation of the current generation across a window of potentials, allowing insight into a species' redox behaviour⁶⁴. As such it can be used to characterise the type and concentration of redox mediators and to probe the mechanism by which the EAM perform EET. Voltammetric methods most suitable for analysing EAM include cyclic voltammetry (CV) and pulse methods such as differential pulse voltammetry (DPV) and square wave voltammetry (SWV). Such techniques are superior to linear sweep voltammetry (LSV), as they can ascertain whether a reaction is reversible- a characteristic necessary for any component involved in EET. In the case of CV, the potential of the working electrode is scanned linearly with time to a set value and then returned to the starting point, with the response current continuously recorded. This generates an I vs E curve, which is usually populated by characteristic peaks corresponding to the activity of an EET mechanism (i.e. recording the positive or negative current occurring during the oxidation or reduction by the electrode of an EAM's cytochrome or shuttle.) The height of these peaks provides insight into the concentration of redox-active compounds such as electron shuttles and cytochromes and the distance between the oxidative and reductive peak helps establish whether a process is diffusion limited

(as with soluble electron shuttles found in MET) or diffusion independent (as with cytochromes found in DET).

The incorporation of voltammetric techniques into electromicrobiology studies has already begun, with Yuan et al.⁴⁰ conducted a range of electrochemical analyses in order to characterize ARB enriched from WWTP in an MFC, ultimately calculating electron transfer rate from scan rate analysis and Laviron theory. Similarly, Carmona-Martinez et al.⁶⁹ used CV to characterise the electron-transfer mechanisms of *Geoalkalibacter subterraneus*, a novel bacterium isolated under potentiostatic conditions from salt plant sediment. Such analyses allow a more quantitative approach to be taken for the characterisation of new and existing EAM.

1.5.3 Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) consists of the application of a sinusoidal AC current at various frequencies to unveil the frequency-dependent behavior of the electrochemical interface. EIS and its derivative methods are used to study the deposition of EAM on the electrode and the formation of interfaces, e.g. biofilms. These electroactive interfaces can be characterised in terms of their electrochemical behavior at various frequencies, thus identifying the main charge transfer processes. Early EIS work on MFCs with single EAM species was reviewed by He and Mansfield⁷⁰, who discussed how EIS can be used to determine MFC internal resistance and the activity of EAM biofilms. They suggested that the effect of redox mediators (exogenous or self-produced) on charge transfer resistance and biofilm development on the electrode can be effectively investigated by EIS. In a more recent study, Babauta and Beyenal⁷¹ analysed *G. sulfurreducens* biofilm grown on a rotating disk electrode

to demonstrate the effect of diffusional limitations on EET, showing that interfacial resistance increased greatly under electron donor-limiting conditions.

For mixed species biofilms, EIS is less informative, as it cannot resolve the contribution of individual species to the overall impedance. In an early study on marine biofilm formation on stainless steel, Faimali et al.⁷² found that the charge carrier density in the passive layer film did not depend on the biofilm. In a more recent review, Dominguez-Benetton et al.⁷³ outlined the limitations of EIS use in a mixed community BES. EIS data can be easily fitted with non-ideal circuit elements, like the constant phase element (CPE). However, meaningful interpretation of EIS data requires quantification of biofilm thickness and of bioreactor dimensionless numbers.

When correctly applied, EIS is a non-destructive analytical method and provides unique information. However, EAM are metabolically-active systems that produce a small current even under non-growth conditions. Therefore, some of the finest EIS techniques cannot be applied to these complex systems. EIS is a global method, thus it returns an overall picture of the electrochemical interface. Local EIS (LEIS), which is based on the use of microelectrodes and measures the current density in solution above a surface feature (e.g., a corrosion pit or a microbial colony) offers much higher definition at the microscale than global EIS, but its application to mixed species biofilms performing EET is largely unreported³. To the best of the author's knowledge, application of LEIS to microbial biofilms has been recently suggested, with no experimental data reported to date⁷³⁻⁷⁴.

At a fundamental level, EIS is a technique which can examine the resistance to charge transfer, R_{CT} . This is very useful in terms of EAM as it can track the R_{CT} in response to electrode colonisation, with a decrease expected to be

seen as an electroactive biofilm forms over time. Furthermore, the R_{CT} can be tracked across a potential window, from cathodic (i.e., electrode as electron donor) to anodic (i.e., electrode as electron acceptor) charge transfer, allowing detection of emerging electron transfer mechanisms which facilitate charge transfer and thus reduce its resistance. The frequency of electron transfer can also be determined, allowing comparison with known microbial electron transfer processes. In addition to making inferences from the raw data, EAM systems can frequently be fit using simple models with two or more time constants, such as a modified Randles cell, where the high frequency constant depends on the material interface and the low frequency constant depends on the metabolic processes. While application of EIS to enrichment studies is currently not widely practiced, it has frequently been reported in the study of sulphate-reducing bacteria in the context of bioMIC and MFCs^{72, 75-76}.

Caution must be exercised when interpreting EIS data for biological systems, owing to their inherent complexity forbidding many of the assumptions which can be made in traditional electrochemistry. The reader is referred to existing in-depth reviews discussing the application of EIS to microbial electrochemical systems^{73, 77}.

1.6 Community profiling

Electroanalysis provides evidence for EAM enrichment in terms of current generation and how it's achieved. However, it cannot resolve EAM from other microorganisms, or provide information about the relative abundance of EAM or give insight into the exact genes being expressed (which could contribute to the development of an overall model for community enrichment). Community profiling via culture-independent techniques such as metagenomics and

metatranscriptomics is an excellent complement of electroanalysis, as it allows unveiling of the complexity of the enriched community (DNA), while giving information on the electron transfer mechanisms (RNA). While still not the gold standard in the field, if community profiling is carried out, it tends to focus on metagenomics rather than metatranscriptomics (Table 1.1) The reader is referred to recent reviews ⁷⁸⁻⁷⁹ on the application of such techniques to environmental microbiology.

1.7 Future perspectives

Despite extensive efforts in electrochemical enrichment, it is likely the majority of EAM diversity is yet to be discovered, with waste water and sediment microbial communities often resulting in rediscovery of well-known EAM such as *Geobacter* ⁸⁰. It is possible this genus may be the most successful and adaptable EAM in nutrient-rich environments. It is also likely that the enrichment methods used failed to select for other EAM present, either due to their slow growth rate or because they are not able to thrive in a diverse consortium. Current results suggest that EAM diversity is very small, indicating that either we have fully explored this small niche or, more likely, that our methods are not broad enough. The small number of species isolated today may be a direct result of certain repeated oversights in terms of starting biological material, medium composition and the enrichment method adopted. A novel roadmap for EAM enrichment is suggested, which focuses on a non-wastewater inoculum source, alternative medium design, and electrochemical and genetic analyses (Figure 1.1.)

Figure 1.1 Proposed streamlined method for EAM enrichment in potentiostat-controlled three-electrode setup.

1.7.1 Starting material

Most enrichments start from WWTP samples, as early studies aimed to increase energy recovery from waste water. However, this limits the inoculum diversity and can result in long enrichment timescales. It is the author's opinion that bioprospecting for EAM should encompass various environments, including those highly contaminated and extreme in nature (in terms of such parameters as temperature, pH, salinity and atmospheric pressure). It is here that we are likely to

observe EET mechanisms unseen before, and one consequence of this effort could be the discovery of novel redox agents of biological origin that can be used in environmentally friendly bioprocesses and biosensors. While it is clear a variety of inoculum choices have been explored, it is proposed here that further expansion of this practice will yield promising results, as the initial studies (Section [1.2.1](#)) indicate a strong argument for differing inocula impacting community structure.

1.7.2 Medium

The role of growth medium has been long overlooked and of the few studies published it is not possible to determine the importance of this parameter in EAM enrichment. Aside from synthetic waste water, few media used in EAM research contain more than one carbon source. The interplay between community members enriched in the presence of multiple low-concentration carbon sources may be more indicative of natural environments and hence could yield synergistic relationships unseen before in EAM research. The work of Hou, Sun and Hu ⁴² highlights the significance of even temporal changes in medium, reporting the presence and absence of *Geobacter* based on the order in which components are added. Furthermore, no ideal medium has been developed, raising questions as to whether such a “mastermix” could exist, or whether this will depend on initial inoculum. The notion of such a gold standard medium being applicable to all starting materials would be contingent upon a relatively high conservation among EAM metabolism, which already diverges considerably among known species. However such musings raise further questions in relation to the impact of growth medium, such as whether it can play a role in selecting different EET mechanisms. A starting point for such a question could be the transcriptomic analysis of EAM such as *Shewanella* spp., who utilise various EET mechanisms, in response to

alternative medium compositions. Even further interesting questions arise when one considers the competition that may occur in the natural environment where both solid and soluble electron acceptors may co-exist. Media compositions designed with this in mind may unearth novel species.

While possibly considered the unglamorous front-end of EAM research, medium composition is a highly important consideration and often overlooked. This oversight could be partly explain the hitherto surprising lack of EAM diversity.

1.7.3 Reactor configuration

As discussed in Section [1.5](#), a relatively limited degree of control is afforded over the enrichment conditions in MFCs and MECs in comparison to a three-electrode set-up. This is apparent mostly in terms of control over applied potential and electrochemical characterisation possible. While optimising MFC and MEC reactor design and performance has its place, the fundamental insight achieved via potentiostat-controlled half-cells may yield beneficial results in the long-term. The study of Pierra et al.'s⁵¹ resulted in an extremely enriched and efficient EAM community, highlighting the benefits of potentiostatic methods vs MFC technology in terms of reducing the broad distribution of community members identified as an issue in Section [1.4.1](#).

As discussed in Section [1.7.2](#), medium composition is likely a bottleneck in unearthing EAM diversity. However the issue of supplying carbon sources found in nature, typically of a low redox potential, may be mitigated somewhat by merely mimicking the potential of the carbon source during cathodic enrichment, where electrons are consumed by the EAM and a negative current is recorded.

While a certain quantity of carbon will need to be present in the medium for cell growth and maintenance, the majority of the electrons themselves can be supplied via the electrode poised at a specific low potential. This may facilitate the mimicry of unknown carbon sources required by fastidious organisms, particularly in extreme environments, and would ideally be executed in high-throughput mode using a multichannel potentiostat or array of power sources across a multitude of potentials. Such configurations could even be miniaturised to screen microorganisms not only in terms of current consumption but also in terms of EET mechanism, with short-term experiments targeting MET. The same mimicry approach can be applied at the anode, with various electron acceptors represented by various positive electrode potentials. On the topic of extreme conditions, the exposure of enrichment cultures to redox potentials outside the normal range used in laboratory studies could result in growth of novel species; however, care must be taken not to abiotically oxidise the cell membrane thereby causing damage.

Aside from potentiostat-controlled three-electrode systems, even more novel devices for enrichment can be applied, such as the high throughput method for the screening and isolation of EAM using electrochromic material recently described by Yuan et al.⁸¹ which ultimately lead to the discovery of a new Gram positive EAM, *Lysinibacillus sphaericus* D-8⁸², a discovery very noteworthy owing to the issues relating to conductivity and EET faced by Gram positive microorganisms due to their thick layer of peptidoglycan comprising the cell wall.

1.7.4 Electrochemical analysis

Electrochemical methods for characterisation of EAM enrichments aim to determine an increase in interface conductivity (EIS) or active charge transfer (CA). Additionally, CV and pulse voltammetry methods provide information on

the microbially-produced mediators and the onset potential for the charge transfer process(es). However, interpretation of results is not straightforward and experiments with cell-free supernatant and different scan rates are needed to extract valuable information regarding the charge transfer mechanism. While there are reports of studies performing comprehensive electrochemical characterisation⁸³⁻⁸⁶, they are in the minority. Further adoption of such an approach by other researchers will facilitate the building of a more comprehensive picture of EAM activities, allowing comparison and conclusions to be drawn between studies.

1.7.5 Community profiling

As apparent from Table 1.1, the majority of metagenomic studies to date engage in 16S rRNA gene profiling, as opposed to whole genome sequencing, in order to identify known members within the community. While frequently a very fruitful approach, it is important to note there may be limitations in the accurate description of relative abundances of different phyla in diverse microbial communities using this technique. Furthermore, in order to reconstruct the genomes of novel EAM and hence increase the number of reference genomes in the databases against which to map reads during metagenomic studies, it is important to increase the number of studies that perform deep-sequencing at the conclusion of their enrichment (as opposed to 16S rRNA gene sequencing). This will benefit future studies in addition to retroactively contributing to the literature, as past studies with unidentified original taxonomic units may be assigned based on these new database depositions. On a related note, conducting a temporal metagenomic survey, that is, at various time points during the course of the enrichment, would aid the development of a model for EAM community evolution,

as it is currently unclear how exactly an EAM community shifts over the course of an enrichment.

Few EAM enrichment studies employ metatranscriptomics, likely due to difficulties handling RNA in addition to cost considerations (Table 1.1). However this technique will be key in gaining further insights into the genes responsible in EAM's for reacting to environmental factors such as redox potential, as seen in Ishii et al.⁴⁵.

Finally, an important point to note is that few EAM studies use metabolomics and proteomics to profile secreted metabolites and characterise relevant proteins, thus representing a significant gap in current research. By analysing such components (e.g. via mass spectrometry) as part of a combined approach to systems analysis, further insight can be gained into which factors have the strongest effect on EAM metabolism, in addition to possibly leading to the identification of novel shuttles and cytochromes involved in EET.

1.7.6 Outlook

In addition to the main points for consideration mentioned here, there are other factors which should be acknowledged as possibly playing an important role in novel EAM enrichment, or lack thereof. For example, as can be seen from Table 1.1, the majority of EAM enrichment studies utilise various forms of carbon as the electrode material (largely due to cost efficiency in MFC implementation), raising the question of whether this practice can explain the repetitive enrichment of previously known EAM. Alternative materials, and modifications thereof, should be considered to direct the evolution of the EAM community. Practical considerations such as larger electrode surface/cell volume ratio could be used to select for DET-based EAM. Likewise, specific EET pathways could be inhibited in

order to select for desired ET mechanisms, e.g., via frequent replacement of media to remove electron shuttles associated with MET⁸⁷. Other engineering and biological factors with a likely taxonomic impact such as batch versus flow conditions and biofilm age should be taken into account. Furthermore, alternative means of reporting results, such as electrochemically-active surface area (versus apparent surface area) and current per unit biomass⁸⁸, may provide a more meaningful contribution in terms of quantifying the efficiency of a consortium or strain.

A key suggestion is that the conventional MFC enrichment style may have reached its limit in terms of novel EAM selection and as such a progression to alternative practices should be considered. By shifting the focus to finding possibly less efficient, though novel, EAM, further knowledge of the fundamental nature of EET and of microorganism/electrode interactions may be possible. Insights gained through this approach are likely to find application in MFC and MEC technology, even if the novel EAM discovered through the course of enrichment are not themselves suitable.

The increased use and combination of the factors discussed throughout this review will signal the transition of EAM research from an application-driven field to a more holistic study of these organisms as a whole.

1.8 Conclusions

As has been summarised in this review, recent attempts to enrich novel EAM have been multifaceted, with studies focusing on starting material, media, reactor configuration, electrochemical analysis using techniques such as CA, CV and EIS and community analysis via metagenomics/metatranscriptomics. However, it bears noting that few studies have combined these disparate elements

into one streamlined approach. While admittedly cumbersome, it is the author's belief that such an undertaking will yield worthwhile results in the enrichment and characterisation of novel EAM.

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Chapter 2: Motivation

2.1 Electrochemically-active microorganisms: why we should look deeper

The importance of researching electrochemically-active microorganisms (EAMs) at first glance is twofold: (1) they represent a highly novel form of respiration which in itself extends our knowledge of the natural world and (2) they have vast practical potential, in terms of biosensors, bioremediation, and wastewater treatment. As intriguing as these organisms are, both from a basic science and engineering vantage point, the research field centred around them seems to have reached an impasse. Has the collective focus on utilising EAMs for wastewater treatment had the unintended effect of blinding us to other questions and applications?

As identified in [Chapter 1](#), the vast majority of studies use wastewater as their source of inoculum and Microbial Fuel Cells/Microbial Electrolysis Cells (MFCs/MECs) as their reactor configuration of choice. While this approach has paid dividends in certain aspects, a redundancy appears to have emerged as the same two genera, *Shewanella* and *Geobacter* repeatedly are enriched in such experiments, with seemingly little room for alternative microbial members to share the spotlight. This thesis seeks to look beyond these conventional approaches, as the work described herein uses alternative starting material, in addition to a hitherto underused reactor style and subsequent characterisation methods.

2.2 Electrochemically-active microorganisms in tropical sediments

Among the underrepresented inocula in EAM studies, tropical sediments are perhaps one of the most overlooked, with the limited number of studies available primarily focusing on mangrove sediments¹⁻². This could in large part be due to the geographical location of many research laboratories in this field. To address the gap, [Chapter 4](#) of this thesis examines an EAM enrichment using two separate inocula of tropical climate origin; namely, urban canal sediments, in addition to the more frequently investigated mangrove sediments, with the hypothesis that these inocula will give rise to alternatives to the common genera isolated in such experiments, namely, *Geobacter* and *Shewanella*. More specifically, it was hypothesised that the physical characteristics of tropical climates, namely warm temperatures and frequent rainfall, may support the evolution of novel microorganisms not present in other regions. Furthermore, sediments can be a reservoir for metals, which are frequently associated with EET. Indeed, metals in the canal sediment of the present work were found in a separate study to be strong drivers of microbial communities³. It was postulated that this effect may extend to driving novel EAM.

2.3 Complementing the engineering approach: the marriage of traditional electrochemistry with microbiology

While the availability of exotic inoculum sources may be limited by geographical constraints, the reactor style of choice is not. [Chapter 3](#) and [Chapter 4](#) of this thesis describe growth of pure culture and mixed communities, respectively, using a three-electrode set-up borrowed from traditional electrochemistry, as opposed to a microbial fuel cell/microbial electrolysis cell. As discussed in depth

in [Chapter 1](#), this approach has considerable benefits over more complicated reactors owing to its streamlined simplicity which removes many of the variables and confounding factors associated with MFCs and MECs, such as limiting cathode potential, proton exchange difficulties and large internal resistances. Furthermore, the set-up builds upon years of fundamental electrochemistry and lends itself to advanced techniques for electrochemical characterisation such as chronoamperometry/chronocoulometry, cyclic voltammetry and electrochemical impedance spectroscopy. These are highly informative techniques to apply to a live culture ⁴, as demonstrated in [Chapter 3](#), and differ from many of the conventional approaches to characterisation of EAM, such as power output, polarization curves and treatment efficiency ⁵.

This three-electrode approach using a carbon felt working electrode was explored during the first stage of the thesis work, via a pure culture study using *Pseudomonas aeruginosa*, which is discussed in [Chapter 3](#). During these experiments we hypothesised that (1) the redox active metabolites of live cultures of *P. aeruginosa* could be electrochemically monitored in real time using a three-electrode setup and (2) the redox-active metabolite *Pseudomonas* quinolone signal (PQS) can act as an antioxidant for microbially-produced pyocyanin. The study was primarily voltammetric in nature (utilising differential pulse voltammetry).

2.4 Beyond the community: examining the roles of individual isolates

In addition to achieving an electrochemical enrichment, the work described herein was concerned with trying to isolate novel EAM in pure culture. As discussed in [Chapter 1](#), there are a limited number of studies which perform community analysis via metagenomics, and in fact even fewer go beyond that step

and attempt to isolate a novel EAM from their enrichment. This work strove to walk the journey from environmental sample to novel isolate. [Chapter 4](#) and [5](#) discuss the isolation procedure and findings in depth, while exploring the hypothesis that the electrochemical signature of individual isolates could be traced back to that of the original enrichment.

In addition to the goal of enriching novel EAM, this work wanted to challenge the current state of the art, where investigations are primarily working with mixed communities. Bioelectrochemical technology need not only be applied to complex inocula, but also to single species, as detailed in [Chapter 3](#) and [5](#). [Chapter 5](#), in particular, addresses the outlook of the field, which holds opportunity to diversify our applications and even uncover extracellular electron transfer capabilities of microbes hitherto thought incapable of such respiration.

In truth, it is likely that the diversity of EAM could require years to uncover, with many environments yet to be explored, e.g. those under extreme conditions such as high pressure which could be home to various benthic EAM. As such, the work described herein only represents the tip of the iceberg in terms of new approaches that can be employed, but nonetheless strives to form the basis of a solid framework for novel EAM enrichment and isolation which can be adapted with relative ease by others working in the field.

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Chapter 3: Voltammetric profiling of redox-active metabolites expressed by *Pseudomonas aeruginosa* for diagnostic purposes

This chapter has been modified from a manuscript that was published in Chemical Communications ². Lucinda Elizabeth Doyle was co-first author, was involved in the preparation of the manuscript, and took part in all experiments discussed in this chapter.

Abstract

The present study optimised an electrochemical platform for subsequent experiments on a mixed community ([Chapter 4](#)). Real-time electrochemical detection of the quorum sensing molecule *Pseudomonas* quinolone signal (PQS) and phenazines in viable *P. aeruginosa* cultures is reported. Chemical deconvolution of the pyocyanin voltammetric signal from that of other phenazines was achieved. An electrochemical interplay between PQS and pyocyanin was observed, suggesting PQS might protect pyocyanin from self-oxidation. The study represents an application of bioelectrochemistry to a microorganism not usually studied in this context. Fast detection of redox-active metabolites expressed by *P. aeruginosa* may find relevance in diagnostic devices.

² Seviour, T. W.; Doyle, L. E.; Lauw, S. J. L.; Hinks, J.; Rice, S.; Nesati, V.; Webster, R. D.; Kjellenberg, S.; Marsili, E., Voltammetric profiling of redox-active metabolites expressed by *Pseudomonas aeruginosa* for diagnostic purposes. *Chemical Communications* **2015**, 51 (18), 3789-92.

3.1 Introduction

Pseudomonas aeruginosa is a disinfectant-tolerant and antibiotic-resistant pathogen¹⁻² that is common in natural and engineered habitats. While not typically thought of in the context of electrochemically-active microorganisms (EAM), it has been demonstrated to achieve extracellular electron transfer (EET), specifically mediated electron transfer, in microbial fuel cells via use of self-produced pyocyanin and other phenazines as redox shuttles³⁻⁴. These phenazines, including pyocyanin⁵, in addition to the putative signalling molecule 2-heptyl-3-hydroxy-4-quinolone (i.e., *Pseudomonas* quinolone signal, or PQS)⁶ contribute to the virulence of *P. aeruginosa*⁷. Pyocyanin can inactivate host proteases and is considered a direct determinant of *P. aeruginosa* virulence⁷⁻⁸. PQS, on the other hand, is thought to be only an indirect determinant of virulence through induction of *lasB* and *rhII*, which encode the LasB elastase and N-butanoyl-L-homoserine lactone synthase (RhII), respectively, thereby disrupting innate and adaptive immune systems^{6,9}.

Pyocyanin and PQS are redox-active, which has implications for the metabolism and extracellular defences of *P. aeruginosa*. Reversible cycling of pyocyanin between redox states at a potential (E) of approximately -200 mV vs. Ag/AgCl produces hydroxyl and superoxide radicals, which can damage epithelial cells¹⁰⁻¹¹. Pyocyanin is also available to *P. aeruginosa* as a redox mediator, to maintain redox-balance under anaerobic conditions and to control the intracellular redox state¹². The bisoxygenated aromatic structure of PQS allows it to act as an antioxidant which increases the tolerance of *P. aeruginosa* to oxidizing disinfectants¹³.

Regulation of pyocyanin and PQS expression by *P. aeruginosa* are interdependent. PQS and its effector gene (PqsE) control the biosynthesis of phenazines, while pyocyanin is itself the terminal-signalling molecule and contributes to upregulation of PQS and PqsE synthesis^{5, 14}. While it is clear that pyocyanin and PQS are linked at the level of genetic regulation, it is also possible that they interact via homogeneous electron transfer mechanisms. Reversible redox cycling of pyocyanin produces reactive oxygen species (ROS) that irreversibly oxidise part of the pyocyanin molecule¹⁵. As an antioxidant¹³, PQS may in fact protect pyocyanin from this self-oxidation.

Voltammetric detection of pyocyanin has been reported. However it is unclear to what extent its voltammetric peak is obscured by the signal of other phenazines. Bukelman et al.¹⁶ described using electrochemical techniques the regulation of pyocyanin production by N-acyl homoserine lactones, another family of quorum sensing molecules. Bellin et al.¹⁷ used a miniaturised sensing platform to spatially resolve the concentrations of phenazines in *P. aeruginosa* colonies. Both assigned the dominant voltammetric signal, at -0.270 and -0.300 mV vs. Ag/AgCl respectively, exclusively to pyocyanin. This contrasts with previous findings that assigned the same peak only partially to pyocyanin³. Electrochemical analysis of PQS has also been reported in an abiotic system at concentrations in the nanomolar range using boron-doped thin-film diamond electrodes¹⁸.

Simultaneous, real-time electrochemical detection of PQS and pyocyanin in growth cultures, however, has not been previously reported. Such detection, along with improved means to resolve the contribution of pyocyanin to the voltammetric signature of the phenazines, is required to further elucidate pyocyanin function and its chemical interplay with PQS. High-resolution detection of *P. aeruginosa* redox

metabolites would help early diagnosis and treatment of sepsis and other life-threatening infections ¹⁹. In addition to addressing these questions, the application of these electrochemical techniques to a pure culture facilitated exploration of this set-up for later use with a mixed species community, in the context of an electrochemical enrichment (discussed in [Chapter 4](#)).

3.2 Experimental procedure

3.2.1 Bacterial growth conditions

Wild-type *P. aeruginosa* strain PAO1 ²⁰ and the $\Delta pqsC + sPqsE$ ⁴ mutant were used. The mutant strain lacked PQS expression but could produce phenazines. Each strain was grown overnight in LB medium at 20 °C under shaking conditions of 150 rpm, to an OD₆₀₀ of 1.0. One millilitre of the culture was added to 9 ml of modified ABTG medium ²¹ in a three-electrode electrochemical cell (EC). Briefly, the medium contained 15.1 mM (NH₄)₂SO₄, 33.7 mM Na₂HPO₄, 22.0 mM KH₂PO₄, 0.05 mM NaCl, 1 mM MgCl₂·6H₂O, 100 µM CaCl₂, 1 µM FeCl₃, and 28 mM glucose (C₆H₁₂O₆). The culture was then grown in the EC for 16-20 h at the specified potential at 30 °C under constant stirring of 500 rpm.

3.2.2 Electrochemical set-up and analyses for viable *P. aeruginosa* cultures

Single chamber ECs with a working volume of 10 ml were operated in a three-electrode configuration on a VSP five channel potentiostat (Bio-Logic, France). ECs were assembled as previously described ²². The counter electrode was a 0.1 mm titanium wire (Sigma-Aldrich, Singapore) which was inserted into glass capillaries and soldered to a copper wire. The working electrode consisted of a 1 x 1 cm x 3.18 mm piece of carbon felt (Alfa Aesar, USA) connected to the potentiostat via titanium wire, a nylon screw and a nut (Small Part, USA). The

resistance of each electrode assembly did not exceed 2.5 ohm. Prior to each experiment, the assembled ECs were sterilised at 121 °C for 20 min. The Ag/AgCl reference electrode (Alpha Analytical, Singapore) was connected to a 3 mm Vycor glass membrane (Alpha Analytical, Singapore) via a salt bridge consisting of 1 M KCl in 1.5% agar.

Chronoamperometry (CA), and differential pulse voltammetry (DPV) were characterised the viable microbial cultures. CA monitored cell growth by recording the current produced. Preliminary experiments on a five-day old *P. aeruginosa* culture showed negligible direct electron transfer following spent medium removal (data not shown). Thus, the current recorded was entirely attributed to mediated electron transfer via microbially-produced redox metabolites. Under these experimental conditions, estimations could be made regarding the growth phase the culture was in at any given time. DPV were used to characterise the resultant metabolites produced during this growth. DPV parameters were: pulse height = 50 mV; pulse width = 0.200 s; step height = 2 mV; step time = 0.400 s; scan rate = 5 mV/s. DPV was performed at the beginning of the experiment and after approximately 20 h, or as specified. In another series of experiments, growth cultures were poised at 100 mV and 400 mV. Control experiments were maintained at open circuit potential, i.e., native growth conditions. DPV was performed at 0, 5, 10, 15 and 20 h.

3.2.3 PQS standard addition and calibration

PAO1 after 20 h of growth at 100 mV was centrifuged at 6000 rpm and filter-sterilised to obtain cell-free supernatant. This was then diluted x 5 in sterile medium and added to a clean EC with a newly prepared carbon felt electrode. DPV was conducted and the peak height at 230 mV noted. Known concentrations of

PQS standard were added (0.25-2 μM) with DPV performed after each addition. The increase in peak height was then plotted versus PQS concentration, allowing extrapolation to find the concentration in the starting supernatant.

3.2.4 H₂O₂ and MPII addition experiments in viable *P. aeruginosa* culture

PAO1 was grown overnight at 400 mV and DPV was performed after 20 h. 10 μM microperoxidase MPII and H₂O₂ concentrations ranging from 1 mM to 5 mM were then added, with DPV performed after each addition.

3.3 Results

3.3.1 Voltammetric detection of PQS and phenazines

P. aeruginosa PAO1 was grown in electrochemical cells poised at various electrode potentials and the current output was recorded (Figure 3.1). The strain produced minimal current at 0 mV and, despite the higher driving force, applying 400 mV did not result in notably higher current than was seen at 200 mV. The electrochemical profile of microbially-produced redox metabolites as a function of time was then determined through DPV. The DPV of *P. aeruginosa* PAO1 grown for 20 h at E = 100 mV vs. Ag/AgCl showed three well-resolved redox peaks at -210 ± 10 , 110 ± 10 and 250 ± 10 mV vs. Ag/AgCl (Figure 3.2A). The peak centred at -210 mV was consistent with that of phenazines detected on carbon electrodes in aqueous solution²³⁻²⁴. The peak centered at 250 mV corresponded to PQS, as verified by the standard addition method. The average PQS concentration in growth cultures was $3.32 \pm 2.04 \mu\text{M}$ (Figure 3.2B).

Figure 3.1 Representative chronoamperometry of PAO1 grown at $E = 0$ mV vs. Ag/AgCl (black), 0.2 V (red) and 0.4 V (blue). pH 7.4, $T = 30^\circ \text{C}$.

The DPV of the *P. aeruginosa* PAO1 grown at 100 mV vs. Ag/AgCl was recorded at $t = 0, 5, 10, 15$ and 20 h (Figure 3.2D). Following a brief lag period, the concentration of both phenazine and PQS increased with time. The DPV of the $\Delta\text{PQSc} + \text{sPqsE}$ mutant which lacked PQS expression did not display the peak at 250 mV after 20 h (Figure 3.2C). The peak centered at 110 mV likely corresponded to a feature of the microbial membrane, or a biomass-adsorbed chemical species, as it did not appear when testing the cell-free filtrate (data not shown). Therefore, this peak was not assigned to any of the *P. aeruginosa* metabolites tested in this work.

Figure 3.2 Voltammetric characterisation of wild type and mutant *P. aeruginosa*. (A) DPV of *P. aeruginosa* PAO1. (B) PQS determination in the supernatant of *P. aeruginosa* PAO1. (C) DPV of *P. aeruginosa* PAO1 (black trace) and its $\Delta PqsE$ mutant (red trace). (D) DPV of *P. aeruginosa* PAO1 at 0 h (black), 5 h (red), 10 h (blue) and 15 h (green) and 20 h (orange) showing the increase of PQS concentration with time. *P. aeruginosa* cultures were grown on carbon felt electrodes for 20 h at $E = 100 \text{ mV vs. Ag/AgCl}$ and 30°C . Scan rate = 5 mV/s .

Although PQS is known to be irreversibly oxidised at $E > 800$ mV on a diamond electrode¹⁸, the effect of mild oxidative potentials on PQS concentration is not known. Here, *P. aeruginosa* was grown on electrodes poised at potentials between -200 mV and 400 mV vs. Ag/AgCl. The PQS concentration was measured based on peak height relative to a standard curve and it decreased as potential increased from 0 to 400 mV vs. Ag/AgCl. The maximum PQS concentration after 20 h of growth was observed at -100 to 0 mV vs. Ag/AgCl (Figure 3.3A and B).

Figure 3.3 Examination of PQS concentration in response to growth at various potentials. (A) DPV of *P. aeruginosa* PAO1 across the potential of the putative PQS peak at $t = 20$ h, $T = 30$ °C, with working electrode poised at -200 mV (green), 100 mV (blue) and 400 mV (black), and open circuit potential (red). (B) Maximum height of putative PQS peak from the DPV of *P. aeruginosa* PAO1 as a function of electrode poisoning potential.

3.3.2 Voltammetric detection of pyocyanin

Reszka et al.¹⁵ demonstrated the irreversible oxidation of pyocyanin by hydrogen peroxide (H_2O_2) in the presence of several peroxidases. This oxidation targeted the phenolate group and is specific for pyocyanin. We therefore postulated that irreversible oxidation by H_2O_2 could allow the contribution of pyocyanin to the voltammetric profile of *P. aeruginosa* to be decoupled from that of other phenazines.

Pyocyanin peak-deconvolution by MPII/ H_2O_2 was undertaken in the presence of cells of the wild-type PAO1 and the $\Delta\text{pqsC} + \text{sPqsE}$ mutant. A large reduction in the phenazine peak was observed in the wild-type and no reduction in the peak was observed in the $\Delta\text{pqsC} + \text{sPqsE}$ (Figure 3.4), consistent with what was observed in accompanying experiments conducted on the cell-free filtrate (data not shown)²⁵.

Figure 3.4 Chemical deconvolution of the pyocyanin signal from that of other phenazines. (A) DPV of *P. aeruginosa* PAO1 growth culture (black) following MPII and H₂O₂ addition to 1 (red) and 10 (blue) mM with baseline correction for the phenazine peak (inset). (B) DPV of *P. aeruginosa* PAO1 Δ pqsC + sPqsE growth culture (black) following MPII and H₂O₂ addition to 10 mM (red). $t = 20$ h, pH 7.4, $T = 30$ °C, $E = 100$ mV vs. Ag/AgCl, 5 mV/s scan rate, carbon felt electrode.

3.3.3 The interplay between PQS and pyocyanin

PQS concentration was determined at five time points (0, 5, 10, 15 and 20 h) at open circuit potential (OCP, i.e. native conditions), and 400 mV (Figure 3.5). The time-dependent accumulation of phenazine at 400 mV vs. Ag/AgCl was not accompanied by an induction of PQS expression (Figure 3.5B). Under OCP conditions, both the phenazine and PQS peak increased with time (Figure 3.5A). The comparison of normalised peak heights for PQS showed that PQS expression and accumulation was slightly higher at 100 mV than under OCP conditions (Figure 3.5C).

As described here, poisoning the electrode at potentials greater than 250 mV vs. Ag/AgCl leads to irreversible PQS oxidization. In contrast, pyocyanin is not irreversibly oxidised at potentials ≤ 400 mV. The net effect of irreversible PQS oxidation on pyocyanin expression by *P. aeruginosa* can therefore be observed by growing cultures at $E > 250$ mV. Pyocyanin concentrations were compared using electrochemical peak deconvolution in cultures grown at $E = 100$ mV and 400 mV vs. Ag/AgCl and OCP. A corresponding decrease in phenazine signal intensity was observed at 400 mV relative to OCP and 100 mV vs. Ag/AgCl. A reduction of 5 % in the phenazine peak following MP11/H₂O₂ addition was observed for cultures grown at 400 mV compared to approximately 50 % for those grown at -200 mV (data not shown)²⁵. Pyocyanin therefore constitutes a very small component of the total phenazines when *P. aeruginosa* is grown at $E > 250$ mV, accounting for the reduction in intensity of the phenazine peak compared to growth at -200 mV.

Figure 3.5 DPV of *P. aeruginosa* PAO1 growth cultures on carbon felt electrodes at (A) open circuit potential (native conditions) and (B) 400 mV. (C) Height of PQS peak normalized to the maximum PQS peak height (at 100 mV), at 0 h (black), 5 h (red), 10 h (blue) and 15 h (green) and 20 h (orange). T = 30 °C. Scan rate = 5 mV/s.

3.4 Discussion

Indications of an interaction between PQS and pyocyanin by homogeneous electron transfer mechanisms become apparent in Figure 3.4, where the Δ pqsC + sPqsE mutant, not expressing PQS, sees minimal input from pyocyanin to the phenazine peak. This is in contrast to the wild type, whose pyocyanin contribution to the phenazine peak is much higher. This indicates that PQS may exert an anti-oxidant effect on pyocyanin, thus protecting it from self-oxidation. It has been shown that PQS can act as an anti-oxidant, due to its bisoxygenated aromatic structure that readily donates electrons to form an oxidized ortho-quinoid ¹³

(chemical structure of PQS available in Appendix 3). Collaborators recorded the electron paramagnetic resonance spectra for the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical with and without the addition of equimolar amounts of PQS. It was found that PQS quenches the DPPH radical, thus providing further evidence of its ability to act as an anti-oxidant. Pyocyanin is susceptible to self-oxidation by hydroxyl radicals generated during redox cycling ¹⁵. Hence, it is possible that PQS mitigates this self-oxidation. The loss of an anti-oxidant like PQS could therefore leave pyocyanin more susceptible to irreversible oxidation, resulting in decreased phenazine peak heights as observed in Figure 3.5.

However the multifaceted role of the phenazines, which includes acting as electron mediator during *P. aeruginosa*'s EET ³⁻⁴, cannot be discounted when interpreting these results. The inverse relationship between pyocyanin concentration and electrode potential could also be related to the requirement for energy gain for the cell. As the working electrode potential decreases, the energy gain for the also cell decreases. This could result in a requirement for higher concentrations of pyocyanin at lower electrode potential, in order to increase extracellular anodic respiration in response to this limitation in terminal electron acceptor. This would provide a short-term survival advantage, albeit at the bioenergetic cost of increased production of phenazines. However the results of the $\Delta pqsC + sPqsE$ mutant indicate the answer may not be this straight forward, and could be a combination of multiple factors.

As demonstrated in this study, bioelectrochemical detection of redox-active metabolites in microbial cultures is sensitive and requires little or no sample preparation. The means to characterise a greater range of *P. aeruginosa* metabolites and their chemical and biological interactions broadens the possibility

for the application of electrochemical sensors in patient diagnostics. Additionally, it increases our understanding of the mechanisms of *P. aeruginosa* virulence. We have provided three examples of the diagnostic possibilities for electrochemical profiling with regards to describing aspects of *P. aeruginosa* metabolism. Further research in this area could expand the range of microbial metabolites that can be detected through electrochemical methods, thus leading to rapid characterization of *P. aeruginosa* infections in healthcare settings.

Furthermore, this study demonstrates the applicability of electrochemistry to live cultures, even when they consist of microorganisms not primarily known for EET capabilities. While only one peak in the voltammetric signature (associated with phenazine) is reported to be associated with electron transfer during respiration¹⁵, the other peaks appear over the course of cell growth and their behaviour can be characterised in response to various conditions, such as oxidative stress. This demonstrates the usefulness of electrochemical techniques in the study of microbes who may even be incapable of EET, so long as they secrete redox-active metabolites during their growth. This study also proves that carbon felt is a suitable working electrode to support a high enough volume of biomass in order to detect a current via chronoamperometry/chronocoulometry, while still being a material upon which it is practical to perform voltammetry.

3.5 Conclusions

Real-time electrochemical detection of the quorum sensing molecule PQS and phenazines in viable *P. aeruginosa* cultures was achieved. The pyocyanin voltammetric signal could be distinguished from that of other phenazines by chemical deconvolution through the use of MPII and H₂O₂. Homogeneous electron transfer between PQS and pyocyanin was indicated, suggesting PQS might protect

pyocyanin from self-oxidation. The study represents an application of bioelectrochemistry to a microorganism not usually studied in this context. Fast detection of redox-active metabolites expressed by *P. aeruginosa* may find relevance in diagnostic devices. From a broader perspective, this study optimised an electrochemical platform for subsequent experiments on a mixed community, which will be described in [Chapter 4](#).

3.6 References

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Appendix 3: Chemical structure of PQS

Reduced form of Pseudomonas Quinolone Signal (PQS) ³

³ Haussler, S.; Becker, T., The pseudomonas quinolone signal (PQS) balances life and death in *Pseudomonas aeruginosa* populations. *PLoS Pathogens* **2008**, 4 (9), e1000166.

Chapter 4: Electrochemical and Genetic Analyses of an Enriched Community from Tropical Sediment

This chapter has been modified from a manuscript that is in press in Journal of Power Sources⁴. Lucinda Elizabeth Doyle was first author, prepared the manuscript, and took part in all experiments discussed in this chapter.

Abstract

Enrichment of electrochemically-active microorganisms (EAM) to date has mostly relied on microbial fuel cells fed with wastewater. This study aimed to enrich novel EAM by exposing tropical sediment, under-reported in the literature, to sustained anodic potentials. Sediments originating from a canal system and a mangrove region of Singapore were enriched separately for EAM. Canal sediment was found to be superior to mangrove in terms of electrogenic activity when evaluated by chronoamperometry. Voltamperometric techniques and electrochemical impedance spectroscopy, performed over a wide range of potentials, characterised extracellular electron transfer over time. The applied potential was found to have an effect on both biofilm conductivity and electrochemical signature. *Geobacter metallireducens* was heavily enriched on the electrodes, as determined by metagenomics and metatranscriptomics. *Chlorobium*, *Clostridium* and *Rhodopseudomonas* were also present. This work outlines a comprehensive methodology of characterising novel EAM from unconventional inocula.

⁴ **Doyle, L. E.**; Yung, P. Y.; Mitra, S. D.; Wuertz, S.; Williams, R. B.; Lauro, F. M.; Marsili, E., Electrochemical and genomic analysis of novel electroactive isolates obtained via potentiostatic enrichment from tropical sediment. *Journal of Power Sources* **2017**, (In Press).

4.1 Introduction

Electrochemically-active microorganisms (EAM) are defined by their ability to electrically interact with an external conductive material (e.g. electrodes in the laboratory and metal oxides in nature) via extracellular electron transfer (EET) ¹⁻². They achieve this typically by coupling internal oxidation of an electron donor to extracellular reduction of an electrode, though the inverse has also been reported where electrons are consumed and soluble acceptors reduced ³. The modes of EET vary, and can largely be grouped into two categories; direct electron transfer (DET), which utilises outer-membrane c-type cytochromes and nanowires, and mediated electron transfer (MET) which utilises soluble electron shuttles either secreted by the microorganism or exogenously added ⁴. The unusual respiratory capability of such microbes to interact with an external conductive material can be exploited to aid energy recovery via microbial fuel cells (MFCs) ⁵, achieve bioelectrosynthesis ⁶, serve as the basis of biosensors ⁷, inhibit microbially-induced corrosion ⁸, while in nature playing a key role in biogeochemical metal cycling ⁹. By far the most widely studied EAM are anode-respiring bacteria (ARB). An advantage of working with ARB is that electrode potential dictates the thermodynamic energy available for growth ¹⁰, as opposed to soluble electron acceptors such as O₂ as is the case for many non-EAM. As such, the supply of this driving force can be carefully controlled by use of a potentiostat, opening the door for applications of many conventional electrochemical techniques to probe these electricity-generating communities.

However in contrast to the wide diversity of microorganisms in nature's arsenal, a comparatively narrow catalogue of EAM has been isolated in pure culture with few novel species fully sequenced. This inconsistency is illustrated by

the domination of the past two decades of EAM research by two genera in particular, namely, *Shewanella* spp.¹¹⁻¹² and *Geobacter* spp.¹³⁻¹⁴ Consequently, there is keen interest in adding to the current diversity of known EAM, an endeavour that has culminated in a plurality of enrichment techniques to isolate these specialised organisms¹⁵. However despite the many approaches available, the bulk of EAM studies focus on consortia enriched in MFCs, biological galvanic cells designed to harness the flow of bacterial current⁵.

While useful for generating power from wastewater, this set-up is not optimised for in-depth electrochemical characterisation of EAM and their subsequent isolation¹⁵. In MFCs, the potential of the anode is not under external control and is allowed to drift over the course of the experiment as the ARB colonise the electrode. This affords diminished control over enrichment conditions and may even select for ARB capable of adapting to a wide range of potentials, possibly ruling out more fastidious organisms that may not be recovered in pure culture otherwise. As a consequence of the focus on MFC reactors, many studies employ wastewater as the inoculum source, a recurring choice which could contribute to the lack of diversity seen in many EAM isolates.

Waste water harbours a remarkable microbial diversity, is easily available and highly reproducible, and serves as the intended substrate of energy-harvesting MFCs from a commercial perspective¹⁶⁻¹⁸. However, it typically contains mesophilic microorganisms adapted to circumneutral pH, in an environment typically devoid of insoluble electron acceptors – likely a key selective pressure for EET. When viewed through this lens, it becomes plausible that by diversifying the inoculum choice, a corresponding diversity could be found in known EAM species. Recent years have seen an increase in such alternative inocula, which have

shown promising results ¹⁹⁻²³, but this approach remains the exception rather than the rule.

This study combined the streamlined approach of a potentiostat-controlled three-electrode set-up with a currently under-explored but promising inoculum source, tropical climate sediment, in order to expand our current understanding of EAM. Due to the greater comprehension of the mechanisms employed by anodic EAM, the current study chose to focus solely on the bioanode as opposed to the biocathode, with enrichments operated under batch conditions at oxidative potentials for 4 months, with stirring omitted to select for DET, as opposed to MET. The electroactivity of enrichments was evaluated using chronoamperometry (CA), while their electrochemical signature was tracked with cyclic voltammetry (CV), and the impedance of the biofilm formed on the electrode was characterised via electrochemical impedance spectroscopy (EIS). At the conclusion of the enrichment, the communities at each potential were revealed using metagenomics and metatranscriptomics.

This pipeline for characterisation addresses a tertiary scope of this study: to provide a comprehensive modern method of characterising EAM on the journey from mixed community to isolate, which can serve as a blueprint for other researchers in the field. The combination of techniques from electrochemistry (CA, CV, and EIS), molecular biology (metagenomics, metatranscriptomics and whole genome sequencing) and conventional microbiology (isolation of pure cultures) form an interdisciplinary approach for EAM enrichment and isolation.

4.2 Experimental

4.2.1 Overview

Sediments from equatorial environments, generally under-represented in the literature, were chosen as inoculum for the enrichment. In the first set of experiments, sediments from a flume originally seeded with sediment from a Singapore residential area canal were chosen as representative of a highly anthropically-affected environment²⁴. In the second set of experiments, sediments from Sungei Buloh Wetland Reserve (sampled ~45 cm deep) in Singapore were chosen as representative of a near-pristine environment.

Sediments were inoculated in bioanode conditions, i.e., without soluble electron acceptors, in potentiostat-controlled electrochemical cells maintained at various electrode potentials. Enrichments were operated under batch conditions without stirring for 3-4 weeks before reinoculation, to select for slow-growing EAM capable of DET. Multiple reinoculation steps followed (totalling four months experimental time). CV and EIS were performed at regular intervals in order to characterise the electron transfer mechanisms and biofilm evolution respectively. CA was conducted continuously to measure the current production.

In the case of the canal sediments, a pre-enrichment step was conducted whereby 7 g of sediment was added to three reactors and poised at 200 mV, 300 mV and 400 mV vs Ag/AgCl respectively for more than 50 d to encourage the growth of EAM present. The current output for 300 and 400 mV was similar. This resulted in only the 200 mV and 400 mV reactors advancing to the next stage, chosen due to the large difference in driving force.

After this pre-enrichment step, the long-term experiment commenced with the transfer of the biofilm on the working electrode of the 200 mV and 400 mV reactors into two sterile reactors. The electrochemical cells were poised at different potentials (Table 4.1).

The pre-enrichment step described above for the canal sediments was not repeated for the mangrove sediments as it was decided the protocol was inadequate since unwashed sediments carry reduced chemicals, such as humic acids, which obfuscate the results of the electrochemical enrichment via the appearance of abiotic oxidation currents in the chronoamperometry and voltammetry. Instead an initial washing step was added to the protocol. Briefly, in an anaerobic chamber 2.5 g of mangrove sediment was added to 30 ml of degassed phosphate buffered saline (PBS), shaken and allowed to settle. This procedure was replicated three times to serve as inoculum for three reactors. Ten millilitres of the supernatant was added to a reactor identical to that described above and topped up with 120 ml of degassed M1 media. Applied potentials were 0 mV, 200 mV and 400 mV.

All experiments were conducted at room temperature with stirring omitted to select for DET rather than MET. To select for aerotolerant microorganisms, nitrogen gas was not continuously purged in the headspace during the enrichment cycles.

4.2.2 Biofilm reinoculation

At defined intervals, the working electrode was subjected to a biofilm transfer conducted in an anaerobic chamber (SHEL LAB, USA), where the biofilm was detached from the carbon felt and reinoculated into a fresh setup. Briefly, the biofilm transfer was achieved by suspension of the working electrode in 10 ml of PBS, vigorous shaking by hand, and scraping of any remaining biofilm using a

sterile 1- μ l inoculating loop. Use of PBS in this manner has previously been reported as a successful method for detaching bacteria ²⁵. The PBS was then added to 120 ml of degassed modified M1 medium (pH 7) present in a new reactor. The medium contained, per litre, 20 mM lactate, 7.5 mM NaOH, 30 mM HEPES, 28.04 mM NH₄Cl, 1.34 mM KCl, 4.35 mM NaH₂PO₄, and 0.68 mM CaCl₂ and was supplemented with trace vitamins and minerals ²⁶.

4.2.3 Electrochemical setup and electroanalysis (enrichment phase)

Reactors consisted of a carbon felt working electrode (1 x 1 x 0.3 cm) (Alfa Aesar, Singapore), a titanium counter electrode (Sigma Aldrich, Singapore), coiled 30 times to increase surface area, and a Ag/AgCl reference electrode (Alpha Analytical, Singapore). All potentials discussed in this study are versus Ag/AgCl. The reference electrode was connected via a 3-mm glass capillary and glass frit as previously described ²⁷. A salt bridge consisting of 1M KCl in 1.5% agar was used. Components were fitted inside a glass serum bottle with a working volume of 130 ml and fitted with a butyl rubber stopper. Both canal and mangrove enrichments were characterised with CA and CV, with canal sediments further characterised using EIS.

During CA, which was maintained continuously, we chose to operate at a potential of 200 and 400 mV vs. Ag/AgCl, to impose a strong electrochemical driving force on the EAM.

For CV, the scan rate was either 1 mV/s or 10 mV/s, as specified, which is slow enough to detect turnover electron transfer (ET) on a 1x1 cm carbon felt electrode. Initial and final potential were set between -0.8 and 0.5 V vs Ag/AgCl to capture all anodic phenomena and the transition between cathodic and anodic

electron transfer. The results of scan rate analysis were inconclusive (data not shown), likely because of the large surface area of carbon felt electrodes.

SPEIS (Staircase Potentio Electrochemical Impedance) parameters were as follows: $E_{\text{initial}} = -0.8 \text{ V}$, $E_{\text{final}} = 0.5 \text{ V}$, potential steps = 25, wait time between steps = 2 m, recording time = every 10 s, scan frequency of $f_i = 100 \text{ kHz}$ to f_f of 50 mHz with 15 points per decade, sinus amplitude = 30 mV, 2 measures per frequency.

Table 4.1 Potentials applied to each reactor during the enrichment from canal sediment. Biofilm reinoculation event occurred after each enrichment cycle [C1-C6]. Switching of applied potential occurred at the start of Phase 2 (which constitutes the majority of the results discussed in Section [4.3](#).)

Enrichment Cycle	Potential (mV)	
	Reactor A	Reactor B
<i>Phase 1</i>		
C1	200	400
C2	200	400
<i>Phase 2</i>		
C3	400	200
C4	400	200
C5	400	200
C6	400	200

4.2.4 DNA and RNA extractions

At the conclusion of the enrichment, DNA and RNA were extracted from the bioelectrodes. DNA was extracted using FastDNA SPIN Kit for Soil (MP Biomedicals, USA). RNA was extracted using ZR Fungal/Bacterial RNA Microprep Kit (Zymo Research, USA).

4.2.5 Library Preparation and Sequencing

(i) DNA Samples

Prior to library preparation, the quality of the DNA samples was assessed on a Bioanalyzer 2100, using a DNA 12000 Chip (Agilent). Sample quantitation was carried out using Invitrogen's Picogreen assay. Library preparation was performed according to Illumina's TruSeq Nano DNA Sample Preparation protocol. The

samples were sheared on a Covaris S220 to ~ 450 bp, following the manufacturer's recommendation, and uniquely tagged with one of Illumina's TruSeq LT DNA barcodes to allow pooling of libraries for sequencing. The finished libraries were quantitated using Invitrogen's Picogreen assay and the average library size was determined on a Bioanalyzer 2100, using a DNA 7500 chip (Agilent). Library concentrations were then normalized to 4 nM and validated by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems), using qPCR primers recommended in Illumina's qPCR protocol, and Illumina's PhiX control library as standard. The libraries were then pooled at equimolar concentrations and sequenced on an Illumina HiSeq 2500 sequencer in rapid mode at a read-length of 150 bp paired-end.

(ii) RNA Samples

Prior to library preparation, the quality of the RNA samples was assessed on a Bioanalyzer 2100, using a RNA 6000 Nano Chip (Agilent). Sample quantitation was carried out using Invitrogen's Ribogreen assay. Library preparation was performed according to Illumina's TruSeq Stranded mRNA protocol with the following modifications: The oligo-dT mRNA purification step was omitted and instead, 200 ng of total RNA were directly added to the Elution2-Frag-Prime step. The PCR amplification step, which selectively enriches for library fragments that have adapters ligated on both ends, was performed according to the manufacturer's recommendation but the number of amplification cycles was reduced to 12. Each library was uniquely tagged with one of Illumina's TruSeq LT RNA barcodes to allow pooling of libraries for sequencing. The finished libraries were quantitated using Invitrogen's Picogreen assay and the average library size was determined on a Bioanalyzer 2100, using a DNA 7500

chip (Agilent). Library concentrations were then normalized to 2 nM and validated by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems), using qPCR primers recommended in Illumina's qPCR protocol, and Illumina's PhiX control library as standard. The libraries were then pooled at equimolar concentrations and sequenced on an Illumina HiSeq 2500 sequencer in rapid mode at a read-length of 100 bp paired-end.

4.2.6 Metagenomic and metatranscriptomic analysis

Metagenomic and metatranscriptomic sequencing datasets were processed as follows: All sequences underwent quality trimming to remove adaptor and low quality base pairs using cutadapt ²⁸. The V4 hypervariable region of the 16S ribosomal RNA gene was identified using RiboTagger ²⁹

A count matrix containing all identified ribotags was exported for plotting. Profiling of the expressed mRNA sequences was achieved by filtering out the rRNA gene sequences from the dataset using SortMeRNA ³⁰, followed by homology search of the remaining sequences against the NCBI-NR database using Diamond vX.X ³¹, and the output was analysed in MEGAN v5.11.3 ³². Mapping of hits to taxonomy in MEGAN was done using the GI to taxonomy March 2015 version, and mapping of GI assignment of KEGG was done using the Feb 2015 version of KEGG database. A count matrix of reads binned to the genus level classification using the lowest common ancestor algorithm in MEGAN was exported for plotting.

4.2.7 Mapping of *Geobacter* genomes

mRNA reads were searched against known *Geobacter* genomes. Reads mapped to *G. sulfurreducens* and *G. metallireducens* with 90 bp alignment length

were plotted and only the top hits were considered. Fragment recruitment plots were generated using fragrecruit (available at <https://github.com/mstrosaker/fragrecruit>).

gDNA and mRNA reads were mapped to the *Geobacter metallireducens* GS15 genome using bowtie2³³, with option “end-to-end” and “very-sensitive”. The alignment output was visualized in Geneious6³⁴.

4.3 Results

4.3.1 Electrochemical enrichment of canal sediment

(i) Chronoamperometry

Three potentials (200 mV, 300 mV and 400 mV) were evaluated, with 300 and 400 mV displaying similar current output during the pre-enrichment stage (Figure 4.1). Following a long lag phase, all the reactors developed a current output, with the maximum current output at 25 d increasing with the applied potential. However, the sediments were inoculated without washing, thus part of the oxidation current observed might have been due to the chemical oxidation of reduced compounds (e.g., humic acids) in the sediments.

During the first enrichment cycle, C1, the current output increased rapidly at 400 mV compared with what was seen at 200 mV, likely due to the greater driving force (Figure 4.2). The biomass on the electrode was subcultured during the log phase of current generation, before output reached steady state, to maximise the enrichment rate in subsequent re-inoculation steps. A long lag phase (approximately 1 week) was seen during the second enrichment cycle, C2. However the reactor poised at 200 mV ultimately produced a higher current, contrary to what would be expected from a strictly thermodynamic standpoint.

Following the switch of the poisoning potential between the two anodic communities, which marks the transition from Phase 1 to Phase 2, the third enrichment cycle, C3, once again showed that a higher current was reached at 200 mV despite an initially strong current output in the 400 mV reactor (Figure 4.2). In fact this inversion of potentials at the outset of Phase 2 saw an inversion of the current trends seen in Phase 1. This is notable as the inversion of potentials took place on communities that had been enriched for three weeks at a single potential. This implies that despite a lengthy enrichment period, the communities still maintained a degree of plasticity that allowed them to respond quickly to a change in environmental conditions in the form of oxidative stress.

In the fourth, fifth and sixth enrichment cycles, the microbial consortia at both potentials show a similar trend, with a superior early current seen at 400 mV. However, the reactor poised at 200 mV reached a very similar current, given sufficient time.

Figure 4.1 CA of reactors during pre-enrichment phase showing a transient period of high current generation (likely abiotic), followed by a sustained (likely biotic) signal.

Figure 4.2 CA of canal sediments grown long-term at 200 and 400 mV. At the end of each enrichment cycle, the biofilm on the electrode was removed and reinoculated in a new reactor. The switch from Phase 1 to Phase 2 was carried out to test the effect of potential on already adapted microbial communities.

(ii) Cyclic voltammetry

Reactors A and B utilise similar EET mechanisms, though to different degrees (Figure 4.3 and Figure 4.4). All CVs display a turnover EET sigmoidal curve with an onset at -400 mV. At approx. 200 mV there is the onset of a second, much less effective EET mechanism. In all reactors a decrease in signal is apparent by the end of the enrichment cycle, likely due to depletion of electron donor/nutrients.

Figure 4.3 CV of Reactor A (Phase 2; 400 mV) during C3 (A); C4 (B); C5 (C) and C6 (D). A turnover sigmoidal curve with an onset at -400 mV is observed. Scan rate = 10 mV/s.

Figure 4.4 CV of Reactor B (Phase 2; 200 mV) during C3 (A); C4 (B); C5 (C) and C6 (D). Similar mechanisms are seen here as in Reactor A but with decreased magnitude, likely due to the lower driving force. Scan rate = 10 mV/s.

(iii) Electrochemical Impedance Spectroscopy

Nyquist plots for both reactors showed that in the case of the 400 mV reactor, the resistance to charge transfer (R_{ct}) decreased following inoculation, and then increased as the biofilm matures (Figure 4.5A). Due to the presence of an electron transfer mechanism at approx. -400 mV, as identified by CV (Figure 4.2), EIS analysis focused on the central potential of the catalytic curve. The smallest R_{ct} corresponded to the highest current output. In Reactor B poised at 200 mV the impedance modulus decreased following inoculation and remained approximately constant along the experiment, consistent with a steady ability to produce current (Figure 4.5B).

Figure 4.5 Nyquist plots of the 400 mV reactor (A) during C5, and the 200 mV reactor (B) during C6. Corresponding current production during CA is included.

4.3.2 Electrochemical enrichment of mangrove sediment

(i) Chronoamperometry

As with the canal sediments, a working electrode potential of 400 mV resulted in an immediate generation in current during E1 that was later overtaken by reactors with a lower applied potential during E2 (Figure 4.6). Interestingly even the lowest potential, 0 mV, eventually gave rise to increased current output. Applying 0 and 200 mV saw similar currents reached during E2, however during E3 and E4 the 0 mV reactor was the most stable current producer. A transient, unsustained, high current was consistently seen in reactors following electroanalysis via CV, DPV and EIS across the enrichment cycles.

Figure 4.6 CA of mangrove sediments over long-term growth at 0, 200 and 400 mV (enrichment cycle C1-C4). At the end of each enrichment cycle, the biofilm on the electrode was removed and reinoculated in a new reactor.

(ii) Cyclic voltammetry

At the conclusion of the first enrichment period, similar electrochemical features were seen in each reactor (Figure 4.7). All appear to be quasi-reversible and diffusion limited, as seen by broad oxidation and reduction peaks that are distant from each other. This is indicative of MET and is quite distinct from the features observed during the canal enrichment (Figure 4.3 and Figure 4.4). These electrochemical features are quite distinct from those in the original enrichment detailed in [Chapter 4](#).

Interestingly, after the second enrichment period (examined using the slower scan rate of 1 mV/s for increased resolution), each reactor was already diverging in terms of its electrochemical signature (Figure 4.8A). The 200 mV reactor (red) has two apparent mechanisms for ET; one with an onset at ~ -400 mV, and one with an onset of ~ -100 mV with closely situated oxidation and reduction peaks, perhaps relating to cytochromes and their lack of diffusion-limitation. The 0 mV reactor (black) seems to also have two mechanisms; a similar mechanism at -400 mV and another at ~ 100 mV. The 400 mV reactor (blue) seems to have one mechanism with an onset potential of ~ -200 mV. This could be a soluble redox mediator, however the peak is quite broad and could correspond to a number of redox-active molecules with similar potentials that hence cannot be easily resolved. The reactors continued to maintain distinct electrochemical signatures during C3 (Figure 4.8B) and C4 (Figure 4.8C and D), with the latter showing the evolution of the signature within a single enrichment cycle.

Figure 4.7 CV comparison of mangrove enrichment during C1 at 85 h and C2 at 502 h. Scan rate = 10 mV/s. There is evidence of poorly reversible diffusion-limited electron transfer.

Figure 4.8 CV comparison during C2 at 592 h (A) and C3 at 797 h (B). Different ET mechanisms are seen in each reactor. Since C4 was the longest enrichment phase, comparisons at two time points are examined: 168 h (A) and 1344 h (B). The community was not yet stable after 3 rounds of biofilm scrapings, as distinct changes are seen between the two graphs. (C and D) Scan rate = 1 mV/s.

4.3.3 Metagenomics/metatranscriptomics (canal enrichment)

Metagenomics and metatranscriptomics of the enriched communities were performed on each electrode at the conclusion of the final enrichment cycle (Figure 4.9 and Appendices 4A and 4B). Genomic DNA reveals a heavy presence of *Geobacter*, particularly on the 400 mV electrode. This trend is mirrored in the metatranscriptome, where *Geobacter* accounted for much of the activity on the electrode. The findings are similar when the data are analysed with RiboTagger using the v4 variable region of the 16s rRNA gene (Figure 4.10). When blasting the ribotags against the NCBI-nr database, Geobacter 1 (83bp) has a top hit (100% identity) to *Geobacter metallireducens* GS-15, and Geobacter 2 (84bp) has a top hit to *Desulfuromonas acetoxidans* DSM684 (99% identity), 98% to *Geobacter lovleyi* SZ, and 98% to *Geobacter metallireducens*.

While MEGAN relies on the whole metagenome and metatranscriptome to generate relative abundances, RiboTagger focuses solely on the v4 region of the 16S rRNA gene. Therefore the output in each case is slightly different and can be influenced by genome size. Due to these differences it is difficult to conclusively state what the total community structure was, however both outputs concur that *Geobacter* was dominant on each electrode, followed by *Chlorobium*, *Clostridium* and *Rhodopseudomonas*.

Figure 4.9 Percentage relative abundance of major microbial genera based on the whole genome/transcriptome using MEGAN. Biofilms on the electrodes were sequenced at the conclusion of Phase 2, i.e., Reactor A poised at 400 mV and Reactor B poised at 200 mV.

Figure 4.10 Percentage relative abundance of major microbial groups based on analysis of the v4 region of the 16S rRNA gene using RiboTagger. Biofilms on the electrodes were sequenced at the conclusion of Phase 2, i.e., Reactor A poised at 400 mV and Reactor B poised at 200 mV.

Based on the high abundance of *Geobacter* on the electrodes, mRNA reads were mapped against known genomes of *Geobacter* species (Figure 4.11), showing greatest similarity with *G. metallireducens*. Notably there are two distinct gaps where the genes are unknown, indicating areas where the genome of the enriched *Geobacter* diverged from the reference genome. Similar gaps are apparent when mRNA and gDNA reads were mapped onto *G. metallireducens* GS-15 using bowtie2 (Figure 4.12.) A *G. metallireducens* genome was recovered with 71% coverage (Appendix 4C), however at this percentage it was not possible to identify the genes of the missing regions.

Figure 4.11 Fragment recruitment plot showing mRNA reads searched against known *Geobacter* genomes, showing percentage identity versus position on genome. Top row: 200 mV enrichment mapped against *G. sulfurreducens* PCA (A), *G. sulfurreducens* KN400 (B) and *G. metallireducens* GS-15 (C). Bottom row: 400 mV enrichment mapped against the same (D, E, F). Greatest similarity seen between the 400 mV electrode and *G. metallireducens* GS-15 (F), as seen in the density of plot. Gaps in genome indicate genetic novelty within genus and are marked with arrows.

Figure 4.12 Coverage map (log scaled) of mRNA reads mapped to *G. metallireducens* GS15 genome. Triangular arrows key: dark red: ncRNA, orange: tRNA, blue: rRNA. Regions not found in the reference genome are indicated in broken rectangles.

4.4 Discussion

4.4.1 Current generation from tropical sediments

This work demonstrates that tropical sediments are not all made equal in terms of electroactivity, as demonstrated in the difference in magnitude between the chronoamperometries of canal- and mangrove-derived communities (Figure 4.2 and Figure 4.6). Despite previous work demonstrating promising results using mangrove sediment ²¹, in the present study the canal enrichment was electroactively superior to the mangrove enrichment. However, more studies are needed to ascertain whether this effect holds true for other canal and mangrove sediments. Nonetheless, the superior performance of the canal enrichment in this case was the key factor in choosing the canal enrichment to proceed for community analysis using metagenomics and metatranscriptomics. Various reasons could contribute to the enhanced current generation seen from the canal enrichment, among them anthropic influences which may have resulted in metal oxides in this sediment, which is a key driving force for the emergence of EET ⁹. This is as opposed to the more pristine environment from which the mangrove sediment was obtained, although diagenetic processes have previously resulted in such metal oxides in this type of sediment ³⁵. However, it is not possible to investigate these possible compositional differences without in-depth profiles of each, which was beyond the scope of this work. Furthermore, it is important to acknowledge that the selective pressures imposed by any enrichment regime will by definition exert a large effect on the original microbial diversity present in the sediment. Therefore, alternative parameters such as medium composition would likely have resulted in different electroactive communities and current output.

The complex dynamic of the current output seen in the canal sediments (Figure 4.2) suggests that there are several phases in the electrochemical enrichment, possibly corresponding to several shifts in the electroactive community or in the microstructure and electroactivity of the anodic biofilm. This long-term experiment shows that high poisoning potential results in rapid current generation but does not seem to give a long-term advantage to the microbial consortia in terms of respiration and current output. One reason for this effect could be the high oxidative stress imposed by applying 400 mV, perhaps resulting in damage to the cells, as *Geobacter* is typically grown at potentials not exceeding 200 mV³⁶⁻³⁷. Indeed, *G. sulfurreducens* has been demonstrated to reach a limiting current at 400 mV, where increases in potential see no further increase in current³⁸. With high current flow to the electrode necessitating high levels of proton removal from the biofilm, pH limitations may have hindered bacterial performance in the long term. They could have had a negative impact both metabolically and in terms of cytochrome redox potential, which is affected by pH according to the Nernst equation³⁹. An alternative reason could be that the high energetic payoff inherent in a 400 mV terminal electron acceptor could see the formation of a thick biofilm which ultimately blocks the electrode once cells enter the decline phase. In any case, it is clear that despite the initial acceleration in electroactivity afforded by the 400 mV electrode, this effect is not sustainable.

The mangrove enrichment chronoamperometry displayed notably different features. The transient, unsustained, high current (Figure 4.6) was consistently seen in reactors following electroanalysis via CV, DPV and EIS across the enrichment cycles and was most likely due to charging of the biofilm during the analysis time whereby the bacteria may be inactive if the electrode is scanning

through a range outside of its preferred redox window. As such, “stored” electrons may be discharged upon return to CA, thus displaying a capacitive behaviour ⁴⁰. This dramatic emission of electrons seen as spikes in current is not particularly evident in the 0 mV reactor, perhaps due to the lower driving force between the bacteria and the working electrode.

The comparison of voltammetric signatures across the two sediment sources at 10 mV/s (Figures 3, 4 and 7) indicate different charge transfer mechanisms and, combined with the CA data, underscores the difference in electroactivity of the two sources. In the case of the canal enrichment, CVs show a turnover EET sigmoidal curve with an onset at -400 mV, sharing similarities with *Geobacter* voltammograms in the literature ⁴¹.

4.4.2 Biofilm conductivity

With the canal enrichment producing such a high current, it was decided to investigate the conductivity of the biofilm using EIS. The increase of impedance in the last two weeks of the 400 mV reactor (during Phase 2) could be due to accumulation of thick biofilm or partial shielding of the electrode due to the presence of dead cells, and is consistent with this reactor’s consistent inability to sustain its high current generation. The time-dependent behavior of R_{ct} is likely related to the accumulation of biomass on the electrode, which decreases the biofilm conductivity, seen most prevalently in the 400 mV reactor. However, the occurrence of damaged biomass at the interface, which decreases metabolic ET, cannot be excluded. Similarly, the accumulation of non-electroactive microorganisms cannot be ruled out and has previously been proposed at high potentials ¹⁰. Notably, the effect of biomass accumulation on the impedance of the sample poised at 200 mV is not evident. This suggests that at low potential there

may be less biofilm growth or that the selected community behaves differently, allowing sustained current generation.

4.4.3 Community analysis

As discussed in Section [4.1](#), *Geobacter* is a common genus associated with EAM enrichment studies. However *G. sulfurreducens* is by far the most widely reported species, with *G. metallireducens* mentioned in notably fewer studies. *G. metallireducens* is primarily studied in the context of acetate degradation ⁴²⁻⁴⁵, so its abundance in the canal enrichment, which used lactate as electron donor, was unexpected. This appears to be the first report of *G. metallireducens* appearing in an enrichment fed with lactate. So far, the species has only been shown to achieve lactate degradation after manipulations in a laboratory setting ⁴⁶. Furthermore, since N₂ gas was not purged continuously in the reactor headspace over the enrichment cycles, it is surprising to see the species, frequently considered an obligate anaerobe, thriving in such an environment. However due to the lack of stirring, it is likely that the biofilm became microaerophilic as it formed, with oxygen scavenging microorganisms removing trace O₂ from the bulk solution.

Clues to the survival of *G. metallireducens* in these non-conventional conditions come from the two distinct gaps in the sequenced genome when compared with the reference strain, GS-15 (Figure 4.11 and Figure 4.12). This indicates a certain degree of novelty, but it is not known if the genes in this region of the enriched strain's genome are responsible for the unexpected growth under these lactate-fed and microaerophilic conditions. While it is unknown whether the enriched novel strain was degrading lactate directly or relying on other community members to convert it to a usable form, this is the first report of the species in a

lactate-based system, co-culture or otherwise. The capacity for this particular strain to oxidise lactate is explored in Chapter 5.

A number of other previously reported EAM were found to be enriched on the electrodes. Focusing on the top three genera after *Geobacter*, as established by both RiboTagger and MEGAN, *Chlorobium*, *Clostridium*, *Rhodopseudomonas* have all previously been associated with anodes⁴⁷⁻⁴⁹. MEGAN identified *Anaeromusa* and *Pelobacter* as being the fifth and sixth most abundant. These genera have also been associated with anodes⁵⁰⁻⁵¹. However *Pelobacter* generally cannot directly reduce an electrode itself, despite its ability to reduce extracellular iron⁵², and therefore may have taken part in syntrophic relationships. A number of these genera may have been responsible for degrading lactate to a more readily oxidisable form for *G. metallireducens*. It is difficult to ascertain what proportion of the current these other EAM may have contributed (due to a masking effect of the high-performing *G. metallireducens*), however their high abundance after the long-term enrichment indicates they were strongly selected.

4.5 Conclusions

In this study, tropical climate sediments were used as inoculum for a long-term anodic enrichment on carbon felt using a three-electrode set-up. Canal sediment was found to be more electroactive than mangrove sediment, implying anthropically-affected environments may harbour more EAM than pristine ones. Voltamperometric techniques and electrochemical impedance spectroscopy demonstrated that applied potential had an effect on both electrochemical signature and biofilm conductivity. Growth at an applied potential of 400 mV vs Ag/AgCl was found to generate a high but unsustainable current, indicating a biological limitation, possibly due to low pH or thick biofilms. There were indications of a

high abundance of an organism with approximately 90% similarity to *G. metallireducens* in the enrichment. However, further work would be required to confidently confirm the species' presence, such as analysis of 16S rRNA and other housekeeping genes, whole genome recovery with a high coverage, or retrieval of an isolate in pure culture. Other EAM were also found to be enriched, including *Chlorobium*, *Clostridium* and *Rhodopseudomonas*, indicating that tropical climate sediment may be host to a wide variety of EAM, and warrants further study.

4.6 References

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Appendix 4A: DNA dataset

SampleID	DNA_s200e	DNA_s400e
Raw reads (R1+R2)	137,151,598	119,334,886
Reads after QC	134,981,477	117,497,711
Reads with NCBI-nr hit	95,635,484	81,505,735
% reads with NCBI-nr hit	70.9	69.4
Reads with hits assigned Bacteria	94,310,652	80,050,428
% hits assigned to Bacteria	98.6	98.2
Reads with hits assigned to Archaea	28,313	33,839
% hits assigned to Archaea	0.03	0.04
Reads with assignment to genus	55,745,567	50,759,812
% hits with assignment to genus	58.3	62.3
Reads mapped to assembly	15,056,353	20,671,537
% reads mapped to assembly	11.2	17.6
Co-assembly statistics		
Number of contigs	92,737	
Total size of contigs (bp)	171,416,956	
Longest contig (bp)	235,118	
Shortest contig (bp)	500	
#contigs > 500bp	92,479	
#contigs >1K	34,322	
#contigs >10K	2,124	
#contigs > 100K	53	
Mean length (bp)	1,848	
N50 (bp)	3,519	

Appendix 4B: RNA dataset

SampleID	RNA_s200e	RNA_s400e
Raw reads (R1+R2)	145,375,952	151,418,908
Reads after QC	144,626,837	150,732,725
rRNA reads (after sortmerna)	141,588,014	144,603,148
% total rRNA reads	97.90	95.93
rRNA_v4	1,816,741	2,489,048
rRNA_v5	1,543,520	1,633,495
rRNA_v6	649,073	526,340
rRNA_v7	637,664	578,161
total rRNA with V assignment	4,646,998	5,227,044
% rRNA reads with V assignment	3.28	3.61
mRNA reads (after sortmerna)	3,038,823	6,129,577
% mRNA/total RNA	2.10	4.07
KO assignment MEGAN (all hits)	114,322	410,194
% KO by MEGAN (all hits)	3.76	6.69

Appendix 4C: Genome reconstructions from metagenomic dataset

Genomic bins derived from co-assembly of reads of s200 and s400. Taxonomic assignment of a bin based on Phylosift⁵. Assignment based on the lowest taxon assigned with marker genes greater than 70% of that taxon. Genome coverage of mapped reads (xCov) is calculated using the following equation: $\# \text{ Reads mapped} * \text{length of read} / \text{bin_size_bp}$. Lengths of gDNA and mRNA reads are 151 and 101 bp, respectively.

bin ID	Phylosift_output	CheckM_Marker lineage	bin_size_bp	Completeness	Contamination	#. gDNA reads mapped s200	#. gDNA reads mapped s400	mapped_gDNA_s200_xC ov	mapped_gDNA_s400_xC ov	#. mRNA reads mapped s200e	#. mRNA reads mapped s400e	mapped mRNA_s200_xCov	mapped mRNA_s400_xCov
binsp1500.8	bin8_Paludibacter_91	k_Bacteria (UID2569)	3,262,063	100	0	446,646	654,726	20.68	30.31	8,409	11,189	0.26	0.35
binsp1500.45	bin45_Acidaminococcaceae_89	o_Selenomonadales (UID1024)	2,040,342	99	1	197,281	1,579	14.60	0.12	5,779	82	0.29	0.00
binsp1500.28	bin28_Betaproteobacteria_82	c_Betaproteobacteria (UID3971)	3,978,382	99	0	2,065	644,867	0.08	24.48	101	1,855	0.00	0.05
binsp1500.1	bin1_Bacteroidetes_72	k_Bacteria (UID2569)	2,321,492	98	2	261,122	935,660	16.98	60.86	3,020	36,696	0.13	1.60
binsp1500.12	bin12_Desulfovibrio_desulfuricans_99	c_Deltaproteobacteria (UID3218)	3,208,117	98	1	865,113	46,040	40.72	2.17	18,650	562	0.59	0.02
binsp1500.16	bin16_Desulfovibrio_desulfuricans_92	c_Deltaproteobacteria (UID3218)	2,934,294	97	1	155,166	554,238	7.98	28.52	3,514	8,005	0.12	0.28
binsp1500.39	bin39_Clostridiales_100	o_Clostridiales (UID1212)	3,785,537	97	6	463,209	5,828	18.48	0.23	94,609	620	2.52	0.02
binsp1500.18	bin18_Elusimicrobia_81	k_Bacteria (UID2495)	1,534,291	97	0	1,481	382,312	0.15	37.63	19	8,141	0.00	0.54
binsp1500.13	bin13_Dechloromonas_89	c_Betaproteobacteria (UID3971)	3,073,615	96	0	6,368	874,107	0.31	42.94	123	14,585	0.00	0.48
binsp1500.32	bin32_Bacteroidetes_chlorobi_85	p_Bacteroidetes (UID2605)	3,556,317	96	2	476,472	23,155	20.23	0.98	20,678	3,536	0.59	0.10
binsp1500.52	bin52_Porphyromonadaceae_86	k_Bacteria (UID2569)	4,406,231	92	8	76,247	273,395	2.61	9.37	1,136	11,603	0.03	0.27
binsp1500.2	bin2_Alphaproteobacteria_85	p_Proteobacteria (UID3214)	1,560,254	92	0	329,466	350,308	31.89	33.90	3,477	5,063	0.23	0.33
binsp1500.46	bin46_Clostridia_84	o_Clostridiales (UID1212)	2,703,475	91	1	504	274,347	0.03	15.32	114	2,211	0.00	0.08
binsp1500.48	bin48_Flavobacteriaceae_100	p_Bacteroidetes (UID2605)	2,286,613	90	1	171,170	1,208	11.30	0.08	9,254	116	0.41	0.01
binsp1500.53	bin53_Clostridiales_incertae_sedis_88	o_Clostridiales (UID1120)	4,171,285	88	5	241,778	20,049	8.75	0.73	3,220	389	0.08	0.01
binsp1500.38	bin38_Oscillospiraceae_100	o_Clostridiales (UID1212)	2,094,606	88	0	254,532	14,750	18.35	1.06	6,341	546	0.31	0.03
binsp1500.49	bin49_Oscillospiraceae_84	o_Clostridiales (UID1212)	2,203,353	87	5	89,823	84,822	6.16	5.81	1,524	1,544	0.07	0.07
binsp1500.22	bin22_Bacteria_100	p_Bacteroidetes (UID2605)	3,398,042	84	2	218,590	350,890	9.71	15.59	14,146	4,101	0.42	0.12
binsp1500.44	bin44_Negativicutes_82	p_Firmicutes (UID1022)	2,727,849	82	3	15,763	272,671	0.87	15.09	530	4,272	0.02	0.16
binsp1500.47	bin47_Clostridiales_90	o_Clostridiales (UID1120)	2,027,795	76	7	1,717	163,472	0.13	12.17	21	1,596	0.00	0.08
binsp1500.31	bin31_Geobacter_metallireducens_88	c_Deltaproteobacteria (UID3216)	2,652,722	71	1	1,275,209	2,218,166	72.59	126.26	26,062	154,159	0.99	5.87
binsp1500.51	bin51_Elusimicrobia_100	k_Bacteria (UID2495)	1,358,078	70	2	37	105,975	0.00	11.78	2	2,840	0.00	0.21

⁵ Darling, A. E.; Jospin, G.; Lowe, E.; Matsen, F. I. V.; Bik, H. M.; Eisen, J. A., PhyloSift: phylogenetic analysis of genomes and metagenomes. *PeerJ* **2014**, 2.

Chapter 5: Electrochemical and Genetic Analyses of Novel Isolates

This chapter has been modified from a manuscript that is in press in Journal of Power Sources ⁶. Lucinda Elizabeth Doyle was first author, prepared the manuscript, and took part in all experiments discussed in this chapter.

Abstract

The aim of the work contained herein was to isolate the novel *Geobacter metallireducens* from the canal enrichment characterised in [Chapter 4](#). However, this proved unsuccessful and two different novel isolates were obtained from the enrichment in pure culture instead. They were identified to be from the genera *Enterobacter* and *Aeromonas*, respectively.

The names proposed were *Enterobacter* sp. EA-1 and *Aeromonas* sp. CL-1. Both isolates were capable of extracellular electron transfer (EET) on carbon felt and screen-printed electrodes without the addition of exogenous redox mediators, while growing in minimal media. Each was able to use acetate and lactate as electron donors. *Enterobacter* sp. EA-1 could perform mediated electron transfer using the soluble redox shuttle 2-hydroxy-1,4-naphthoquinone (HNQ), indicating it may have availed of this mode of EET during the initial enrichment.

⁶ Doyle, L. E.; Yung, P. Y.; Mitra, S. D.; Wuertz, S.; Williams, R. B.; Lauro, F. M.; Marsili, E., Electrochemical and genomic analysis of novel electroactive isolates obtained via potentiostatic enrichment from tropical sediment. *Journal of Power Sources* **2017**, (In Press).

5.1 Introduction

Many studies on electrochemically-active microorganisms (EAM) conclude at the end of an enrichment period and do not go beyond this primary phase to actually isolate novel strains in pure culture ¹⁻⁵. The incorporation of this secondary phase in EAM research has been identified as a key step needed to improve our understanding of EAM and their application ⁶. In addition to establishing the electrochemical and genetic response to an anodic enrichment as described in [Chapter 4](#), a subsequent goal of the current study was to isolate and sequence pure cultures of novel EAM in order to add new strains to the existing repertoire of known EAM. Based on the prominent enrichment of *Geobacter metallireducens* in the primary phase of this study, screening using NBFC plates ⁷ was used and all experiments were conducted under anaerobic conditions.

To efficiently screen isolates for electroactivity, the emerging technology of screen-printed electrodes (SPEs) was employed, while comparing the absolute electroactivity of isolates by chronocoulometry. This pipeline for characterisation addressed a tertiary scope of this study, namely, to provide a comprehensive modern method of characterising EAM on the journey from mixed community to isolate, which can serve as a blueprint for other researchers in the field. The combination of techniques applied across [Chapter 4](#) and [5](#) included electrochemistry (chronoamperometry/coulometry, voltammetry and electrochemical impedance spectroscopy), molecular biology (metagenomics, metatranscriptomics and whole genome sequencing) and conventional microbiology (isolation of pure cultures). This formed an interdisciplinary approach for EAM enrichment and isolation.

5.2 Experimental

5.2.1 Isolation procedure

Due to the abundance of *G. metallireducens* in the enrichments, which represents the first report of the species in a lactate-fed system (co-culture or otherwise), the isolation approach used was optimised for *Geobacter* growth. The original enrichments, which had been stored in glycerol at -80°C, were streaked onto NBFC agar plates, containing, per L, 20 mM sodium acetate, 56 mM ferric citrate, 1 mM L-cysteine, 23.81 mM NaHCO₃, 12.74 mM KCl, 9.35 mM NH₄Cl, 1.44 mM NaH₂PO₄, 0.11 mM CaCl₂·2H₂O and 0.32 mM MgSO₄·7H₂O and supplemented with trace vitamins and minerals. Where specified, the isolates were grown in liquid NBFC, which did not contain agar. All handling and incubations were performed in the anaerobic chamber at 25°C.

5.2.2 Electrochemical setup and electroanalysis

Rather than tracking the evolution of current with time, the absolute electroactivity was measured by evaluating the charge passed by Day 7 and Day 14 using chronocoulometry⁸⁻⁹ which measures the integral of current over time. Reactors were poised at 400 mV vs. Ag/AgCl, unless otherwise specified. Cyclic voltammetry (CV) was conducted at 1 mV/s to observe turnover electron transfer in the absence of diffusional limitations, and at 10 mV/s to highlight quasi-reversible redox couples in the system. Reactor configurations and medium composition took two formats, detailed in 5.2.2.1 and 5.2.2.2.

(i) Carbon felt working electrode

Reactors consisted of a carbon felt working electrode (1x1x0.3 cm) (Alfa Aesar, Singapore), a titanium counter electrode (Sigma Aldrich, Singapore), coiled

30 times to increase surface area, and a Ag/AgCl reference electrode (Alpha Analytical, Singapore). The reference electrode was connected via a 3-mm glass capillary and glass frit as previously described ¹⁰. A salt bridge consisting of 1M KCl in 1.5% agar was used. Components were fitted inside a glass serum bottle with working volume of 130 millilitre fitted with a butyl rubber stopper. Reactors were stirred at 150 RPM to increase mass transfer and N₂ was purged continuously in the headspace to maintain anaerobicity. All reactors were inoculated in biological replicates from single colonies grown anaerobically on NBFC plates as described in 2.1. Reactors contained degassed modified M1 media (pH 7) which consisted of, per L, 20 mM acetate, 7.5 mM NaOH, 30 mM HEPES, 28.04 mM NH₄Cl, 1.34 mM KCl, 4.35 mM NaH₂PO₄, 0.68 mM CaCl₂ and was supplemented with trace vitamins and minerals ¹¹.

(ii) Screen-printed electrodes

Where specified, isolates were also characterised for EET and use of an exogenous shuttle using disposable screen-printed electrodes (SPEs). SPEs are convenient tools for screening microorganisms when there is no need to retrieve substantial biomass (in which case carbon felt is the better choice due to the greater surface area).

Model C110 (DropSens, Spain) was used in experiments, consisting of carbon working electrode (diameter 4 mm), carbon counter electrode and silver pseudoreference electrode. Methacrylate batch cells (DropSens, Spain) with a working volume of 6 millilitres were used.

Isolates were grown for 1 week under anaerobic conditions with the medium described in 2.2.1 with the modification of 20 mM lactate instead of acetate as the electron donor, and the addition of 40 mM fumarate as electron

acceptor. After growth, cells were spun down and washed with fresh media to remove any soluble electron acceptor. Cells were reinoculated into the batch cells described above but with the exclusion of a fumarate as electron acceptor. After 48h of growth at 400 mV vs. Ag, 50 mM of 2-hydroxy-1,4-naphthoquinone (HNQ) was added as soluble electron shuttle (chemical structure of HNQ is available in Appendix 5B).

5.2.3 DNA extraction and sequencing

DNA was extracted from liquid cultures grown using the fumarate-based media detailed in 2.2.2 using the Wizard Genomic DNA Purification Kit (Promega, USA) and sequenced using the MinION SpotON Flow Cell Mk I (R9.4) (Oxford Nanopore, UK). For phylogenetic analysis, The 16S rRNA gene sequences of the isolates were amplified by polymerase chain reaction (PCR) with bacteria-specific primer pair 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' ACGGTTACCTTGTTACGACTT 3')²¹, using genomic DNA as template. Polymerase Chain Reaction (PCR) was carried out in a 50 µl reaction with 1x HF buffer (Thermo Fisher Scientific, USA), 200 µM dNTP, 0.2 µM each primer, and 0.02 U/µl Phusion DNA Polymerase (Thermo Fisher Scientific, USA) to ensure high fidelity amplification of the hypervariable region of the 16S rRNA gene. The PCR conditions were initial denaturation for 30 s at 98 °C; 25 cycles of 10 s at 98 °C, 30 s at 58 °C, and 2 min at 72 °C; and final extension for 5 min at 72 °C. An aliquot of the PCR products was separated by agarose gel electrophoresis, stained with ethidium bromide, and visualised by UV illumination using the Gel Doc XR+ system (Bio-Rad, USA) to detect product size and homogeneity. The PCR products were then purified using PureLink PCR purification kit (Invitrogen,

USA) with the final elution in sterile water and sequenced by capillary electrophoresis (AITbiotech, Singapore).

5.2.4 Identification of 16S rRNA gene sequence and phylogenetic analysis

The 16S rRNA gene sequences obtained by capillary electrophoresis were trimmed to remove low quality sequences, followed by merging the end of the reads through assembly. Sequences were checked for chimeras using DECIPHER v2.0 ²² prior to homology search against the NCBI 16S Bacterial/Archaeal database using BLASTN 2.6.0 ¹². To prepare the phylogenetic tree, multiple sequence alignment and tree construction was done using Geneious 6.0 ²³. UPGMA tree on 16S rRNA gene sequences from genomic DNA of the isolates was constructed with Jukes-Cantor distance model and bootstrap analysis was performed (1000 replication). Support threshold was set at 50%. Sequences were uploaded to NCBI GenBank and are available under the accession numbers KY497447 (*Aeromonas* sp. CL-1) and KY497448 (*Enterobacter* sp. EA-1).

5.3 Results

5.3.1 Isolate identification and electrochemical characterisation

Two isolates were obtained in pure culture from the original enrichment, found with greatest similarity to *Enterobacter* sp. FY-07 and *Aeromonas veronii*, respectively, when sequenced using the MinION. The names proposed are *Enterobacter* sp. EA-16 and *Aeromonas* sp. CL-1. A phylogenetic tree based on the 16S rRNA gene

Both isolates were capable of EET on carbon felt using acetate as electron donor (Figure 5.1). After 7 days, the isolates were comparable in terms of

electroactivity, but after 14 days *Enterobacter* sp. EA-16 had generated more charge than *Aeromonas* sp. CL-1.

Figure 5.1 Comparison of total charge passed after 7 and 14 days for *Enterobacter* sp. EA-16 and *Aeromonas* sp. CL-1 when grown on 20 mM acetate M1. Average of two biological replicates. Error bars refer to range.

Similar mechanisms of EET are used by both isolates after 14 days in acetate-based medium (Figure 5.2). Broad peaks demonstrated diffusion limitation, indicating mediated electron transfer. Diffusion limitation was also suggested by the drift in peak potential as the scan rate increased from 1 to 10 mV/s. The distance between the oxidation and reduction peaks indicated only quasi-reversible electron transfer (ET). These electrochemical features are quite distinct from that which had been seen in the original enrichment detailed in [Chapter 4](#).

Figure 5.2 CV of *Enterobacter* sp. EA-1 (A) and *Aeromonas* sp. CL-1 (B) after 14 days. Two scan rates (1 mV/s and 10 mV/s) are shown for each isolate.

Both isolates were also capable of coupling the degradation of lactate with EET on screen-printed electrodes (Figure 5.3). *Enterobacter* sp. EA-1 was found to be capable of using the soluble mediator HNQ, whereas *Aeromonas* sp. CL-1 was not.

Figure 5.3 Comparison of current production by *Enterobacter* sp. EA-16 (red trace) and *Aeromonas* CL-1 (green trace) showing ability to utilise lactate. 50 mM HNQ added after 48 h on SPE. While more variability is observed in the *Enterobacter* strain, there is a clear distinction between the two isolates abilities to avail of a soluble electron shuttle. Average of two biological replicates. Error bars refer to range.

When grown in NBFC liquid, *Enterobacter* sp. EA-1 appeared to be coated in a black material, which turned orange upon exposure to trace oxygen (Figure 5.4). Similarly, the strain produced black colonies when grown on NBFC plates. This black material in both cases was likely ferrous iron, reduced from ferric iron.

Figure 5.4 Growth of *Enterobacter* sp. EA-1 in NBFC liquid, showing coating in probable reduced iron (left). The white colour of the microorganism can be seen more clearly underneath during later growth (right). Note that there is no electrode in the centre of this serum bottle; the dense mass in the middle is the microorganism.

5.3.2 Electrochemical regeneration of -80°C glycerol stocks

Since *G. metallireducens* was not obtained in pure culture through use of NBFC plates, we hypothesised that it could only be isolated from the electrode itself, however regenerating the enriched 200 mV and 400 mV consortia from the stored -80°C glycerol stocks failed to recover the current. The 200 mV community appeared to never regenerate, while the 400 mV community was electroactive, but far less than was seen in [Chapter 4](#). Feeding with lactate did not have an effect, while 50% medium change had a minor impact on current (Figure 5.5).

Figure 5.5 Current production during regeneration of the 200 mV and 400 mV enriched consortia from [Chapter 4](#).

Cyclic voltammograms of the regenerated 400 mV community at 90 h and 160 h (Figure 5.6), showed an electrochemical signature similar to what had been seen in the original enrichment ([Chapter 4](#)), but present to a decreased degree. A catalytic wave is apparent at 90 h (Figure 5.6A), indicating there is turnover ET. At 160 h there this catalytic wave has given way to typical duck shaped voltammetry (Figure 5.6B), indicating non-turnover conditions due to exhaustion of electron donor/nutrient. The oxidation and reduction peaks present at both 1 mV/s and 10 mV/s demonstrate reversible ET, as seen by the small distance between oxidation and reduction peaks (Figure 5.6B). Furthermore, the peaks centre on the same potential at each scan rate, indicating there is no diffusion limitation, i.e. a film process consistent with direct electron transfer.

Figure 5.6 CV of the regenerated 400 mV community at 90 h (A) and 160 h (B) at two different scan rates (1 and 10 mV/s).

5.4 Discussion

5.4.1 *Enterobacter* and *Aeromonas*: unusual suspects?

While *Enterobacter* and *Aeromonas* were not found in notable numbers in the original enrichment described in [Chapter 4](#), members of these genera have previously been found to be capable of EET¹³⁻¹⁴. Here they were active enough to remain present across all six cycles of the original enrichment, even on an electrode dominated by *G. metallireducens*.

Both isolates could degrade acetate and lactate, which explains their survival in both the original enrichment and the acetate-based isolation protocol. In terms of mechanisms of EET, only *Enterobacter* EA-1 was capable of using the soluble electron shuttle HNQ. This indicates that the *Aeromonas* CL-1 may rely more on direct electron transfer, whereas the *Enterobacter* EA-1 may have taken advantage of mediated electron transfer during the original enrichment by using soluble mediators secreted by other community members. However there is also

evidence of *Enterobacter* EA-1 being able to employ direct electron transfer, as seen in Figure 5.4 where it appears coated in reduced iron under strictly anaerobic conditions which has previously been reported for other electroactive members of the genus ¹⁵. Notably, *Enterobacter* sp. FY-07 (the strain with closest similarity to the isolate) is a known overproducer of bacterial cellulose ¹⁶ which may account for the dense nature of the growth (Figure 5.4).

5.4.2 The case of the missing *Geobacter*

Despite the very high abundance of *G. metallireducens* in the original enrichments described in [Chapter 4](#), it was not successfully isolated during the course of this study. It is possible this outcome is the latest casualty of “the great plate count anomaly” ¹⁷ where culturable microorganisms are estimated at 1% ¹⁸⁻¹⁹.

A host of other possibilities also remain, such as the consortium being negatively affected by storage as -80 glycerol stocks, however this may have only been the case for the consortium originally enriched at 200 mV. The 400 mV consortium appears to be still active, albeit to a far lesser degree than before (Figure 5.5 and Figure 5.6). Furthermore the voltammetry in Figure 5.6 displays an electrochemical signature indicative of *Geobacter* ²⁰.

One possible reason for not achieving isolation is that this particular novel strain of *G. metallireducens* may require a co-culture in order to survive, thus prohibiting it from being obtained in pure culture using techniques available. An alternative, admittedly controversial, possible reason is that the novel strain of *G. metallireducens* had lost its ability to grow on acetate over the course of the original enrichment, and therefore did not respond to the isolation protocols.

In an effort to rescue the current, a 50% medium change was the most effective; though only a minimal increase in current resulted (Figure 5.5). While

such a remedy has been reported in EAM research, it has had mixed results⁶. One possibility for this reduced magnitude in current is *G. metallireducens* losing some of its electroactivity over time in a laboratory environment. This leads to a final consideration; the isolation protocol may have benefited from the use of an insoluble iron gel as electron acceptor, as opposed to soluble iron (III) citrate, as was used. However prohibitive timescales involved in the growth of *G. metallireducens* in this manner ruled out this avenue.

5.5 Conclusions

Attempts were made to isolate in pure culture a novel strain of *G. metallireducens* originally enriched on lactate by electrochemical means from canal sediments. Instead the isolation protocols employed (using acetate) resulted in the cultivation of two novel isolates from the genera *Enterobacter* and *Aeromonas*, respectively. The names *Enterobacter* sp. EA-1 and *Aeromonas* sp. CL-1 were proposed. In order for their novelty with respect to reference genomes to be fully examined and quantified, it would be useful to conduct whole genome sequencing and alignment, beyond the 16S rRNA gene-based phylogenetic analysis conducted here. Both isolates were capable of EET in defined media without exogenous redox mediators on carbon felt and screen-printed electrodes. Each was able to use acetate and lactate as electron donors. *Enterobacter* sp. EA-1 could perform mediated electron transfer using the soluble redox shuttle 2-hydroxy-1,4-naphthoquinone (HNQ), indicating it may have availed of this mode of EET during the initial enrichment. However, indications of direct electron transfer were present for both isolates. The results highlight the survival of weaker EAM on electrodes even in the presence of high performers such as *G.*

metallireducens. The findings also underscore the difficulties in isolating fastidious microorganisms in pure culture.

5.6 References

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Appendix 5A: Phylogenetic tree of isolates

Phylogenetic tree of the isolates showing bootstrap support at each node from 1,000 bootstrap replicates. Aero = *Aeromonas* sp. CL1, Entero = *Enterobacter* sp. EA-1.

Appendix 5B: Chemical structure of HNQ

Chemical structure of 2-hydroxy-1,4-naphthoquinone (HNQ) ⁷

⁷ Woo, S. B.; Kim, D. Y., Enantioselective Michael addition of 2-hydroxy-1,4-naphthoquinones to nitroalkenes catalyzed by binaphthyl-derived organocatalysts. *Beilstein Journal of Organic Chemistry* **2012**, 8, 699-704.

Chapter 6: Conclusions

The work described in this thesis was conducted with the primary aim to enrich novel electrochemically-active microorganisms (EAM) from tropical climate sediments. The secondary goal was to isolate such strains in pure culture and to subsequently sequence them. The tertiary aim was to provide a framework that other researchers in the field can follow when characterising such an enrichment on the journey from complex environmental sample to novel isolate. The work had three main findings: (1) a three-electrode set-up provided an excellent platform for characterising EAM, both in pure culture and in an enrichment, (2) a novel *G. metallireducens* strain unexpectedly thrived in a mixed species community fed with lactate, and (3) two novel electroactive isolates, belonging to the genera *Enterobacter* and *Aeromonas*, respectively, were obtained in pure culture from the original enrichment. This chapter summarises the approach taken to reach these goals, the main conclusions that can be drawn from the work, in addition to recommendations for future work in the area.

6.1 Characterising EAM using a three-electrode set-up

The approach taken to address these goals began with the choice of an alternative reactor configuration. As discussed in [Chapter 1](#), many studies on EAM employ microbial fuel cells (MFCs). We hypothesised that by using the more streamlined three-electrode set-up used in conventional electrochemistry, we could maintain better control over enrichment conditions. Additionally we would have access to a greater range of techniques for probing the nascent electroactive

biofilm, such as chronoamperometry, voltammetry and electrochemical impedance spectroscopy.

As the goal of the work was to ultimately isolate and characterise a pure culture of a novel EAM, it was necessary to confirm that such a reactor configuration would be suitable for this purpose. As such, a study was undertaken to voltammetrically monitor the redox-active metabolites of *P. aeruginosa*. The findings of that particular work are discussed in [Chapter 3](#). The system was found to be robust enough to dynamically monitor the production of pyocyanin and the *Pseudomonas* quinolone signal, in addition to tracking current production with time.

These findings demonstrate that there is a viable alternative to the commonly used MFC-style reactors often cited in the literature. By expanding the range of techniques for monitoring EAM to include chronoamperometry, cyclic voltammetry and electrochemical impedance spectroscopy as the norm rather than the exception, greater insight into the fundamental nature of these microorganisms can be gained. This knowledge can then be applied in MFCs, biosensors and other emerging devices. Furthermore, the findings highlight that electrochemistry can be a very useful tool for studying even weak EAM, such as *Pseudomonas*.

6.2 Tropical climate sediment as inoculum source

With the reactor configuration decided upon, the next stage was to choose the inoculum source for the enrichment study. Due to the under-representation in the literature of tropical climate sediments as environmental samples for EAM studies, such sediments from Singapore were selected. As discussed in [Chapter 4](#), sediments from two distinct regions were chosen: canal sediments from an urban waterway and mangrove sediments from a wetland reserve. The enriched

community resulting from the canal sediments was found be the more electroactive of the two, and was selected for metagenomic and metatranscriptomic analyses. *G. metallireducens* was heavily enriched on the electrodes, challenging the perception that this species typically will not thrive in a lactate-fed environment, even in co-culture. The strain was found to be novel when mapped against the genome of *G. metallireducens* GS-15, with marked gaps where no reference sequence could be found, which may provide clues as to why the strain could survive under conditions that were not strictly anaerobic.

These findings highlight the importance of expanding the choice of inoculum source beyond usual suspects such as waste water. The enrichment of a novel strain of *G. metallireducens* reaffirms the promise of tropical climate sediments as a reservoir of EAM.

6.3 Challenges and rewards of isolating EAM in pure culture

With the enriched community having a high abundance of a novel *G. metallireducens* strain, protocols for the growth of this species were followed with the goal of obtaining a pure culture. While this was unsuccessful (due to numerous possibilities discussed in Chapter [5](#)), two alternative novel EAM were purified instead. The names proposed were *Enterobacter* sp. EA-1 and *Aeromonas* sp. CL-1. Both isolates were capable of extracellular electron transfer (EET) when tested on carbon felt working electrodes and screen printed electrodes.

These findings underscore the need to include isolation of novel strains in pure culture as part of the normal approach of studies in the field. High current producers such as *G. metallireducens* can often “mask” electroactive behaviour of weaker EAM, both in terms of electrochemical signature and in terms of abundance in metagenomic data. However our study demonstrates that weaker

EAM can survive across multiple enrichment cycles even on an electrode dominated by *Geobacter*.

6.4 Recommendations for future work

This work is bookended by the characterisation of three weakly electroactive microorganisms; neither *Pseudomonas*, *Enterobacter* nor *Aeromonas* can rival the electrogenic abilities of *Geobacter*. However, as addressed in a recent commentary on the field ¹, the concept of what constitutes the definition of an EAM is ever-shifting, even after more than 100 years of research. Such debate highlights the need for an enhanced understanding of how and why these weak EAM engage in EET. Future work on this topic should clarify where such EAM fit into the broader picture of the field. Do they share any similarities with the stalwarts *Geobacter* and *Shewanella*, in terms of pathways for EET and motivations for doing so? Furthermore, why do they persist across enrichments on electrodes where they seemingly have such low abundance and current production?

Expanding upon this, the findings of this thesis highlight that electrochemistry can be a very useful tool for characterising weak EAM, as was demonstrated in the *P. aeruginosa* study of [Chapter 3](#). Fundamental questions about the secretion and interaction of redox-active compounds in response to oxidative stress were answered by use of voltammetry. For future work it is important to note that, strictly speaking, a microorganism does not even need to be capable of EET in order to qualify for a three-electrode set-up. The secretion of redox-active metabolites is the minimum requirement for a voltammetric study, which opens the door for an entirely new array of microorganisms that can benefit from the bioelectrochemical toolbox.

Even broader questions can be considered using this platform, including the interactions between EAM and mineral matrices, such as those containing iron. The electrochemical real-time monitoring of bacterial-induced metal reduction can help bridge the gap between engineered devices and the way these microorganisms actually function in nature. The complementation of traditional plate-based research with real-time online monitoring of bacterial respiration is an avenue that could provide fresh insights to the field, from a mechanistic point of view. This is most interesting in relation to weak EAM and the advantage EET may afford them in their natural environment.

It is hoped that the electrochemical and genetic characterisation of an enriched community dominated by a novel *G. metallireducens* strain and the subsequent purification of *Enterobacter* and *Aeromonas* isolates described in this thesis can provide a framework for other researchers in the field embarking upon a study to find the next EAM.

6.5 References

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